

AD _____

Award Number: W81XWH-03D000H

TITLE: Víði • { { { á * ÁÓ[, c@l@asq; ÁÓ^caÁá } a@ * Á ÁÓ[, c@l ÁÓd[* ^} ÉQ•^} • a@^ Á
T^ce cae ÁÓ[} ^ÁS^• Á } •

PRINCIPAL INVESTIGATOR: þó[|^Áp@^•[]

CONTRACTING ORGANIZATION: Qáááæ ÁW} á^• Á
ÁQáááæ Á [|á ÉP ÁÍ GEGÁ

REPORT DATE: Jæ^ ^ ÁGFG

TYPE OF REPORT: Annual Ú^ { { á^

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

*Form Approved
OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE (DD-MM-YYYY) 01-01-2012		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 15 DEC 2008 - 14 DEC 2011	
4. TITLE AND SUBTITLE Transforming Growth Factor Beta Signaling in Growth of Estrogen-Insensitive Metastatic Bone Lesions		5a. CONTRACT NUMBER			
		5b. GRANT NUMBER W81XWH-09-1-0003			
		5c. PROGRAM ELEMENT NUMBER			
6. AUTHOR(S) Nicole Nickerson E-Mail: nknicker@indiana.edu		5d. PROJECT NUMBER			
		5e. TASK NUMBER			
		5f. WORK UNIT NUMBER			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Indiana University Indianapolis, IN 46202		8. PERFORMING ORGANIZATION REPORT NUMBER			
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSOR/MONITOR'S ACRONYM(S)			
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)			
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT While many women with breast cancer will use estrogen receptor therapeutics such as fulvestrant, resistance is nearly inevitable. Recently, it has been noted that the epidermal growth factor receptor (EGFR) family plays a vital role in metastasis to bone; also cross signaling with the transforming growth factor receptor type II (TGFBR2) has been noted to increase levels of destructive environmental cytokines. Therefore we are examining the role of EGFR/TGFBR2 cross talk in bone metastatic breast cancer. The study of these pathways are important to metastatic breast cancer, as they have been known to increase levels of the destructive cytokine parathyroid hormone related protein (PTHRP), which causes increased osteolysis within the bone environment. To elucidate the connection between these two vital receptors may lead to further progression in therapeutics for those women with EGFR positive tumors.					
15. SUBJECT TERMS Transforming Growth Factor Beta, Breast Cancer, Bone Metastasis, Epidermal Growth Factor Receptor, Parathyroid Hormone Related Protein					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 99	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	6
Reportable Outcomes.....	7
Conclusion.....	7
References.....	8
Appendices.....	8

INTRODUCTION

Nearly 80% of breast cancers will eventually metastasize to the bone, causing destructive and painful osteolytic lesions (1). Many of these women will have gone through a series of anti-estrogen treatments. General first line antiestrogen therapy is tamoxifen, which acts as an antagonist with partial agonist characteristics on the estrogen receptor α (ER α) (2, 3); though the majority of women will eventually become resistant to this therapy within 5 years time. Second line therapy thereafter is fulvestrant, which acts as a true ER α antagonist, though ultimately many women will develop resistance to this therapy as well (4-6). The majority of women resistant to fulvestrant will then eventually develop metastases to the bone, and currently there are no available pharmacological therapies for treatment of these bone lesions. We have developed a fulvestrant-resistant MCF7 breast cancer cell line (MCF7-F), which has decreased levels of the estrogen receptor α (ER α) as well as increased expression of the transforming growth factor beta type II receptor (TGF β R2) (7). We have recently adopted direct intratibial injections in our lab for delivery of cells to the bones of mice (8, 9). This method has allowed us to examine the growth of MCF7 cells within the bone microenvironment. Based upon this data and the increased expression of TGF β R2, a hypothesis was formed where the TGF β R2 pathway plays a critical role in metastasis of estrogen-insensitive breast cancer cells to the bone.

The TGF β pathway is a dichotomy in its actions in the cellular environment. In normal cells, TGF β regulates cellular homeostasis and acts to regulate proliferation. Neoplastic cells are able to overcome this TGF β regulation, and frequently use the pathway instead for uncontrolled growth and invasion (10). Another family of receptors often involved in breast cancer metastasis to bone is the epidermal growth factor receptor (EGFR). The EGFR has been linked with increased growth, motility, and proliferation of cancer cells; increased EGFR expression has also been connected to antiestrogen resistance in breast cancers (7, 11). A major connection between the TGF β R2 and the EGFR is the parathyroid hormone related protein (PTHRP). PTHRP is a well-known player in breast cancer metastasis, as it stimulates activation of the destructive osteoclast cells within the bone environment (12). Considering the vicious osteolytic cycle that occurs within bone, metastatic cancer cells secrete large quantities of PTHRP and TGF β to activate osteoclast destruction in the bone (13). TGF β released from the bone will stimulate the TGF β R2 on the surface of the cancer cell, stimulating further proliferation. The cycle continues causing an accelerated breakdown of bone as well as a large increase in tumor size. For the EGFR, it is well known that downstream activation will turn on the PTHRP P3 promoter, further increasing levels of circulating PTHRP (12). We have recently found a possible connection between the EGFR and TGF β R2 signaling, which would lead to a new and interesting view on why many cancers are able to overcome the usual path of TGF β inhibition.

BODY

Task 1 was to examine the ability of MCF7-F cells to grow in bone *in vivo*.

Task 1 was previously completed and reported in the 2009-2010 annual report. We examined the ability of MCF7-F cells to grow in bone simultaneously with human mesenchymal stromal cells (hMSC). We observed that the combination of MCF7-F with hMSC cells caused an increase in osteolytic lesion formation over either cell line injected alone (Figure 1). This finding that MCF7-F cells form destructive lesions with co-injection of hMSC cells suggests that surrounding stroma within the bone plays a vital role in lesion formation after cancer cell metastasis. Task 1 is complete.

Task 2 was to examine the ability of MCF7-F cells to form osteolytic lesions and influence osteoblast to osteoclast signaling *in vivo*. Upon completion of the MCF7-F/hMSC experiments from Task 1, mice were sacrificed and hind limbs removed for histological analysis. Histological sections were prepared and stained for tartrate resistant acid-phosphatase (TRAP) for the detection of active osteoclasts, and also stained for hematoxylin and eosin (H&E). With TRAP staining, an increase in osteoclast counts was seen in sections with both MCF7-F/hMSC cells in comparison to sections with MCF7-F or hMSC cells alone (Figure 2). To investigate if this increase in osteoclast activation is directly from the MCF7-F cells, co-culture experiments were performed with osteoclast precursor cells. Osteoclast precursor cells were primed for 3 days with receptor activator of nuclear factor kappa-B ligand (RANKL), a key factor found on osteoblast cells, and is also involved in osteoclast activation (14-16). The invasive MDA-MB-231 breast cancer cell line, MCF7 cells, or MCF7-F cells were then incubated with the primed osteoclast precursors, followed by TRAP staining in culture. MCF7-F cell co-culture increased the number of TRAP positive osteoclasts as seen in Figure 3. These data suggest the MCF7-F cells may have a more invasive phenotype than wild-type MCF7 cells. We were unable to obtain sufficient cells to perform Pit assays on dentin slices, and so this assay was not performed. The remainder of Task 2 is complete.

Task 3 was to examine the cooperation between EGFR and TGF β signaling in breast cancer cell lines. In order to examine the cooperation between EGFR and TGF β in the MCF7 cell lines, an initial stable MDA-MB-231 cell line was produced with a lentiviral shRNA to EGFR (shEGFR). The shEGFR line showed a 70% decrease in the EGFR compared to vector control cells (Figure 4A). Since parathyroid hormone related protein (PTHRP) is a vital player in the osteolytic cycle, we investigated if there was a change in PTHRP levels in the shEGFR line. shEGFR cells show a significant basal decrease in PTHRP levels compared to vector control. Also, after TGF β treatment, shEGFR cells continue to show a decrease in PTHRP (Figure 4B). Collectively, these data suggest there is a connection between EGFR/TGF β signaling and PTHRP expression in osteolytic destruction.

In order to proceed efficiently to complete tasks for this grant as well as complete the PhD in a timely manner, the MDA-MB-231 shEGFR line was used for continued work. Ongoing experiments include production of a shEGFR line in MCF7-F cells. Since both osteoblasts in the bone microenvironment as well as cancer cells harbor the EGFR, this suggests that EGFR ligands participate in both an autocrine and paracrine manner in the metastatic bone environment. Both paracrine and autocrine EGFR signaling were inhibited with a neutralizing amphiregulin antibody, PAR34, whereas shRNA to the EGFR was used to specifically block autocrine signaling in MDA-231 cells.

Breast cancer metastasis to bone was modeled in female athymic nude mice with intratibial inoculation of MDA-231 cells, and cancer cell-bone marrow co-cultures. EGFR knockdown, but not PAR34 treatment, decreased osteoclasts formed *in vitro*, reduced osteolytic lesion tumor volume, increased survivorship *in vivo*, and resulted in decreased MDA-231 growth in the fat pad. These results are currently in press with PLoS One, and may be found in the appendix, as well as publication 1 in Reportable Outcomes.

As the EGFR has a number of active ligands, we are investigating the role for a number of these specific ligands in the cooperation of EGFR and TGF β signaling. We have created a number of stable shRNA MDA-231 cell lines using a lentiviral packaging system (17, 18) and

shRNAs to EGFR ligands Amphiregulin, HB-EGF, and TGF α . We have also created stable MDA-231 cell lines that overexpress the same ligands, Amphiregulin, HB-EGF, and TGF α . As expected, we observed decreased levels of PTHrP in the shRNA lines and an increase in PTHrP expression in the overexpression lines as seen in Figure 5. Interestingly, invasion was inhibited in both the AREG shRNA and AREG overexpressing line, while overexpression of TGF α caused a significant increase in invasion (Fig 6).

We then injected both the shRNA and overexpressing cell lines into the mammary fat pad of female athymic nude mice. As expected, the shRNA lines increased survival and created smaller tumors by both mass and volume (Fig XX). Simultaneously, the overexpressing lines caused significant decreases in survival as well as larger tumors (Fig XX). Interestingly, *in vitro* it appears that TGF α stimulates more aggressive behavior. In contrast, AREG tumors grew more rapidly than other overexpressing lines.

Together, these results suggest TGF α leads to more invasive behavior, where as AREG leads to larger, more rapidly growing tumors. To defend these findings, we are creating cell lines using the shRNA to AREG or TGF α , and overexpressing the opposite ligand. By doing so, we hope to allow AREG and TGF α to be dominant without competition from the opposing ligand. We are also creating a double knockdown line of both AREG and TGF α inhibition, in hope to nearly completely inhibit signaling by either ligand. After characterization of these new lines, we will inject them into the mammary fat pads of athymic nude mice to observe their tumorigenic capabilities. From these experiments, we expect TGF α overexpression will cause more motile, invasive cells that will create fast crowing, large tumors. We expect that AREG overexpression will create modestly more invasive, motile cells, though not as drastic as the TGF α dominant line. We expect these results to be submitted for publication in May 2012.

Task 4 was to investigate involvement of miRNAs in metastasis and formation of osteolytic lesions. We were unable to initiate experiments for Task 4 due to time constraints.

KEY RESEARCH AND TRAINING ACCOMPLISHMENTS

- Provided evidence that MCF7-F cells are capable of growing osteolytic lesions within mouse bones with support of stromal hMSC cells.
- Demonstrated this combination of MCF7-F/hMSC cells stimulate an increase in osteoclast activation within the bone microenvironment relative to the parental MCF7 line.
- Training in production of Lentiviral constructs as well as subsequent generation of stable cell lines.
- Found that autocrine inhibition of the EGFR is more vital to reducing growth of metastatic breast cancer in the bone than inhibition of receptor signaling in the micro environment.

REPORTABLE OUTCOMES

Manuscripts

1. **Nickerson NK**, Mohammad KS, Gilmore JL, Crismore E, Bruzzaniti A, Guise TA, Foley J. Decreased Autocrine EGFR Signaling in Metastatic Breast Cancer Cells Inhibits Tumor Growth in Bone and Mammary Fat Pad. In press. PLoS One.
2. **Nickerson NK**, Gilmore JL, Allen KT, Riese DJ 2nd, Nephew KP, Foley J (2011). EGFR-Ligand Signaling in Breast Cancer Metastasis: Recurring Developmental Themes. *Breast Cancer - Carcinogenesis, Cell Growth and Signaling Pathways*, Mehmet Gunduz and Esra Gunduz (Ed.), ISBN: 978-953-307-714-7, InTech, Available from:
<http://www.intechopen.com/articles/show/title/egfr-ligand-signaling-in-breast-cancer-metastasis-recurring-developmental-themes>
3. Foley J, **Nickerson NK**, Nam S, Allen KT, Gilmore JL, Nephew KP, Riese DJ 2nd. EGFR Signaling in Breast Cancer: Bad to the Bone. *Semin Cell Dev Biol*. 2010 Dec; 21(9): 951-60.

Degrees

Completion of a PhD in Pharmacology will occur in May 2012.

Poster Presentations

- 2011 Breast Cancer Cell Epidermal Growth Factor Signaling and Osteolysis
(Poster presentation). Congressionally Directed Medical Research Programs, Era of Hope Conference, Orlando, FL.
- 2009 Involvement of Amphiregulin-EGFR Signaling in Breast Cancer Induced Osteolysis
(Poster presentation). Gordon Conference: Bones & Teeth, Biddeford, ME.

Personnel Receiving pay from this effort: Nicole Nickerson

CONCLUSION

Greater than 50% of women with breast cancer will eventually develop metastases to the bone. There is a current lack of pharmacological treatments for these painful bone lesions, requiring ongoing research to produce new therapeutics. In the bone of these patients, a destructive cycle occurs whereby increased expression levels of PTHrP cause increases in the levels of bone destruction. We have noted a possible connection between the pro-oncogenic response of cancer cells to the TGFBR2 and the EGFR; whereby activation of the EGFR may be causing increased expression of the TGFBR2, which in turn may considerably increase available levels of PTHrP. We have also examined how MCF7-F cells interact with the bone microenvironment through co-injection with hMSC cells. These connections are of considerable importance to metastatic breast cancer patients, and may eventually offer a new insight to pharmacological intervention for the TGFBR2 as well as treatment involving the surrounding stromal cells.

REFERENCES

1. L. D. Castel *et al.*, *J Pain Symptom Manage* **34**, 380 (Oct, 2007).
2. C. K. Osborne, S. A. Fuqua, *Breast Cancer Res Treat* **32**, 49 (1994).
3. M. A. Gallo, D. Kaufman, *Semin Oncol* **24**, S1 (Feb, 1997).
4. D. J. DeFriend *et al.*, *Br J Cancer* **70**, 204 (Aug, 1994).
5. S. E. Jones, *Semin Oncol* **30**, 14 (Oct, 2003).
6. C. Morris, A. Wakeling, *Endocr Relat Cancer* **9**, 267 (Dec, 2002).
7. M. Fan *et al.*, *Cancer Res* **66**, 11954 (Dec 15, 2006).
8. O. Berlin *et al.*, *Cancer Res* **53**, 4890 (Oct 15, 1993).
9. J. Zhang *et al.*, *J Clin Invest* **107**, 1235 (May, 2001).
10. J. Massague, *Cell* **134**, 215 (Jul 25, 2008).
11. R. A. McClelland *et al.*, *Endocrinology* **142**, 2776 (Jul, 2001).
12. J. L. Gilmore *et al.*, *Breast Cancer Res Treat* **110**, 493 (Aug, 2008).
13. L. A. Kingsley, P. G. Fournier, J. M. Chirgwin, T. A. Guise, *Mol Cancer Ther* **6**, 2609 (Oct, 2007).
14. R. J. Thomas *et al.*, *Endocrinology* **140**, 4451 (Oct, 1999).
15. D. L. Lacey *et al.*, *Cell* **93**, 165 (Apr 17, 1998).
16. H. Yasuda *et al.*, *Proc Natl Acad Sci U S A* **95**, 3597 (Mar 31, 1998).
17. T. Dull *et al.*, *J Virol* **72**, 8463 (Nov, 1998).
18. J. Moffat *et al.*, *Cell* **124**, 1283 (Mar 24, 2006).

APPENDICES

Reprints:

1. **Nickerson NK**, Mohammad KS, Gilmore JL, Crismore E, Bruzzaniti A, Guise TA, Foley J. Decreased Autocrine EGFR Signaling in Metastatic Breast Cancer Cells Inhibits Tumor Growth in Bone and Mammary Fat Pad. In press. PLoS One.
2. **Nickerson NK**, Gilmore JL, Allen KT, Riese DJ 2nd, Nephew KP, Foley J (2011). EGFR-Ligand Signaling in Breast Cancer Metastasis: Recurring Developmental Themes. *Breast Cancer - Carcinogenesis, Cell Growth and Signaling Pathways*, Mehmet Gunduz and Esra Gunduz (Ed.), ISBN: 978-953-307-714-7, InTech, Available from:
<http://www.intechopen.com/articles/show/title/egfr-ligand-signaling-in-breast-cancer-metastasis-recurring-developmental-themes>
3. Foley J, **Nickerson NK**, Nam S, Allen KT, Gilmore JL, Nephew KP, Riese DJ 2nd. EGFR Signaling in Breast Cancer: Bad to the Bone. *Semin Cell Dev Biol*. 2010 Dec; 21(9): 951-60.

SUPPORTING DATA

Figure 1: Intratibial injection of MCF7-F and hMSC cells

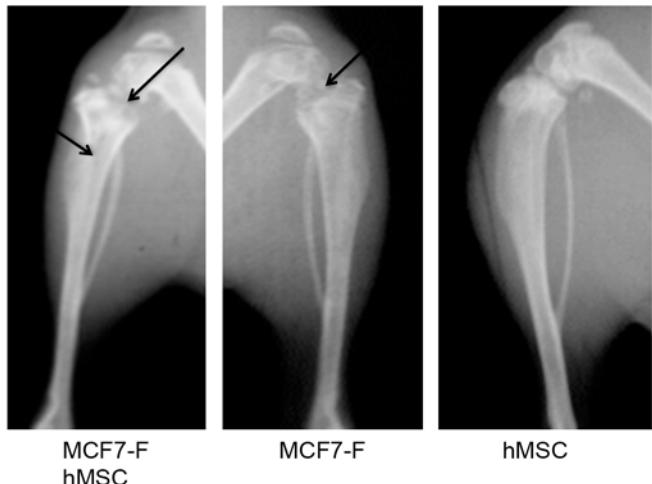


Figure 1: 4-5 week old female athymic nude mice were intratibially injected with MCF7-F, hMSC, or MCF7-F with hMSC cells. Lesions were monitored weekly with X-ray.

Figure 2: TRAP staining of MCF7-F/hMSC injected mouse legs

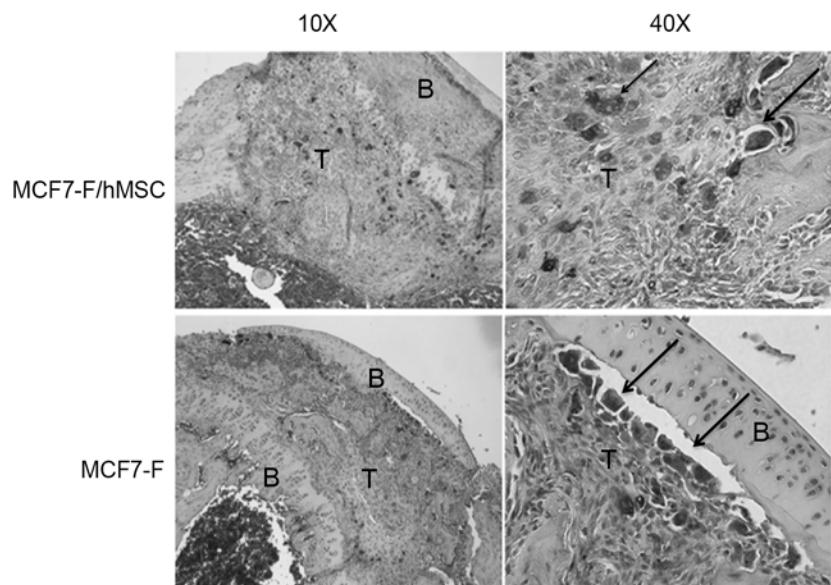


Figure 2: Combination MCF7-F/hMSC cell injections increase osteoclast activation.
Representative TRAP staining from MCF7-F/hMSC or MCF7-F injected animals. Arrows denote positive TRAP stained osteoclasts. B = Bone, T=Tumour.

Figure 3: Co-culture of osteoclast precursors with breast cancer cell lines

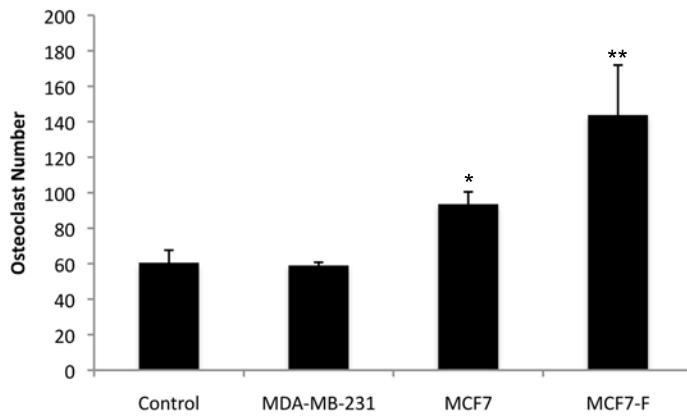


Figure 3: Breast cancer cells stimulate osteoclast maturation. MDA-MB-231, MCF7, or MCF7-F cells were co-cultured with osteoblast pre-cursor cells followed by TRAP staining. Both MCF7 and MCF7-F cells caused an increase in active osteoclasts in culture. * $P<0.05$, ** $P<0.001$.

Figure 4: Knockdown of the EGFR results in a decrease in PTHrP levels

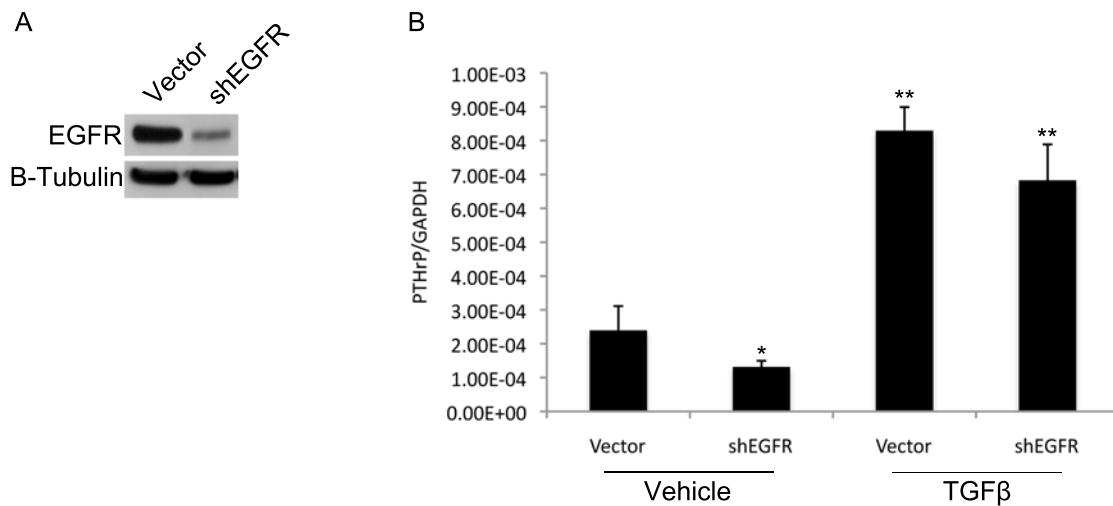


Figure 4: MDA-MB-231 cells were induced with lentiviral shEGFR. A) Representative western analysis of EGFR knockdown in the shEGFR cell line versus Vector control. B) Quantitative PCR analysis of PTHrP levels in Vehicle or TGF β treated Vector or shEGFR cells.

Figure 5: PTHrP expression in EGFR ligand modified stable cell lines

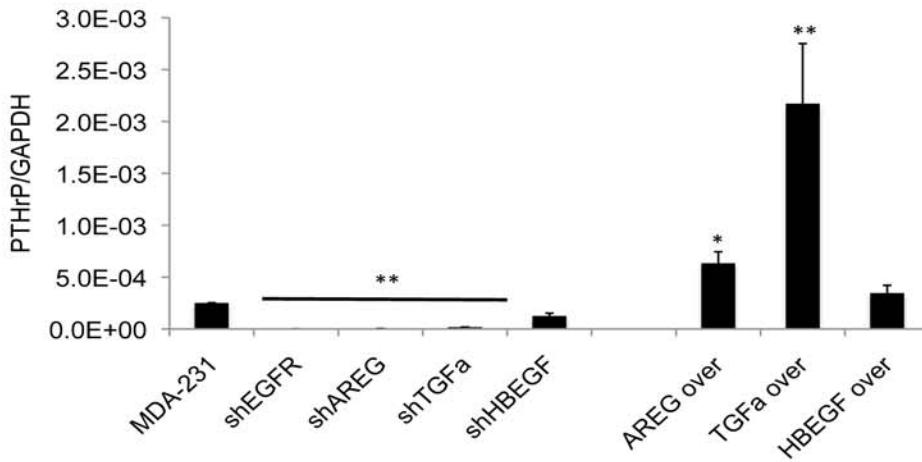


Figure 5: Basal mRNA levels of PTHrP were measured by qRT-PCR in all shRNA or overexpressing cell lines
Significant differences were observed in the shEGFR, AREG, and TGFa cell lines.

* p<0.05, ** p<0.01.

Figure 6: Modifications to AREG or TGFa cause changes in cellular invasion

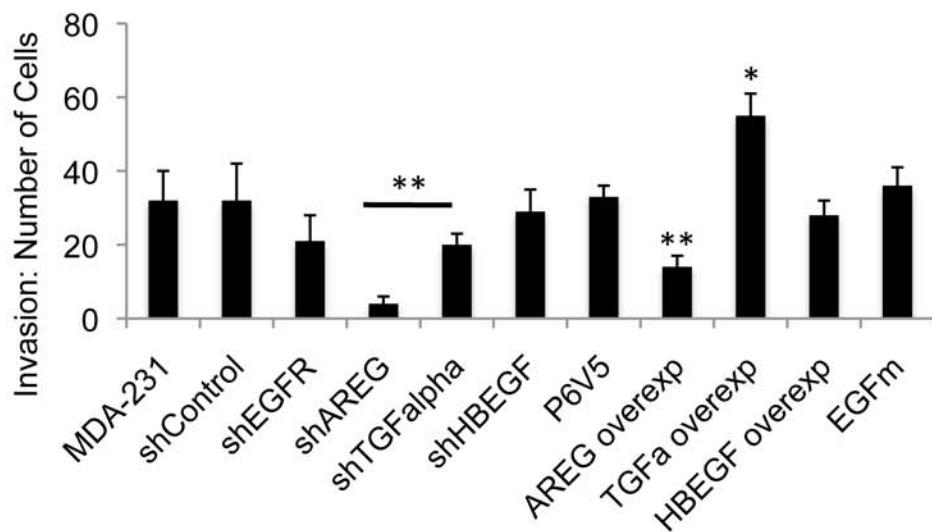


Figure 6: Cells were allowed to invade through matrigel coated BD Transwell invasion chambers for 24 hours before staining the invading cells. Significant changes were observed in both the shRNA and overexpression lines of AREG and TGFa.

* p<0.05, **p<0.01.

Figure 7: In vivo growth and survival of animals with mammary fat pad tumors

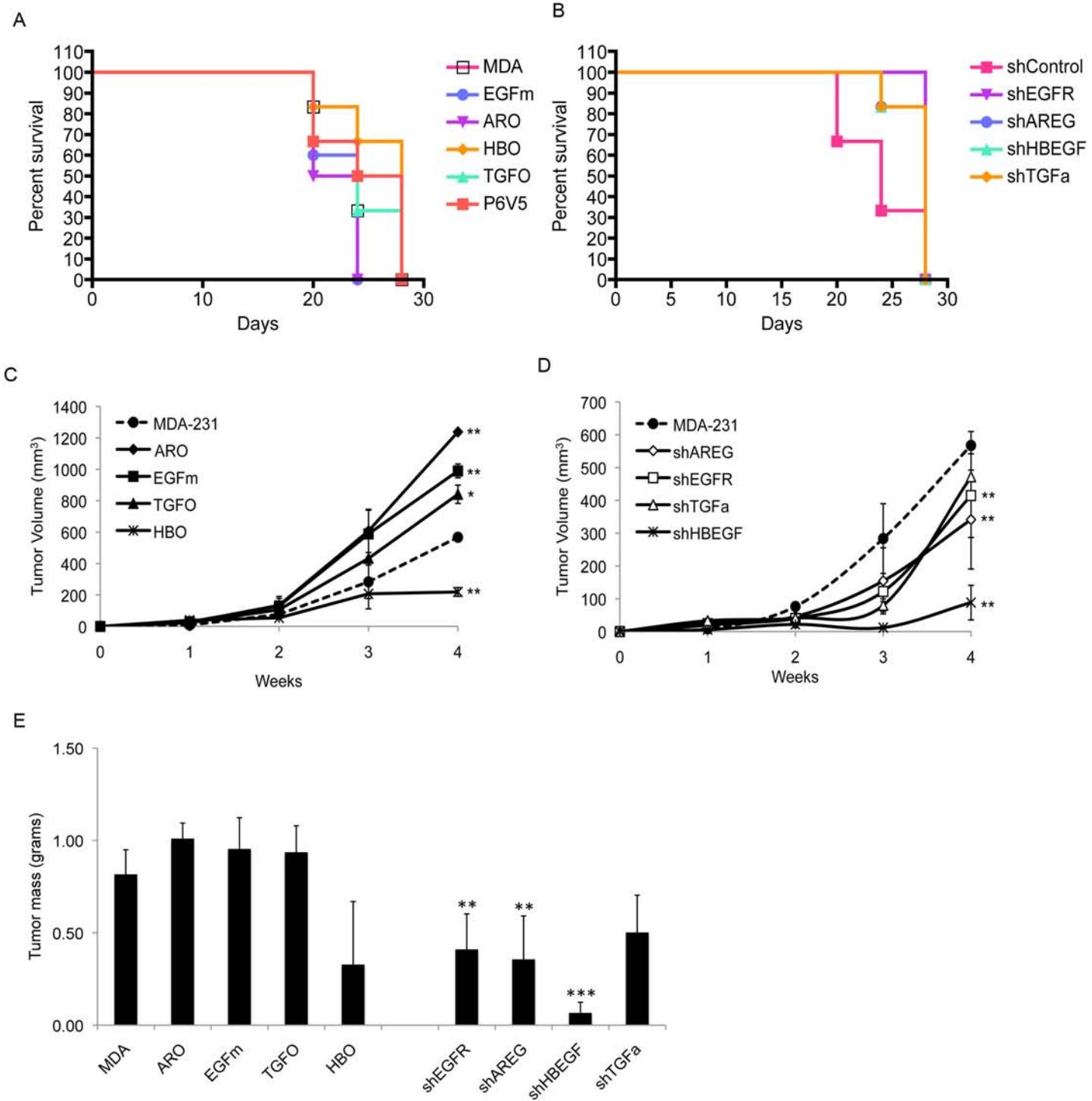


Figure 7: Injection of shRNA and overexpressing cell lines into the mammary fat pad. (A) Ligand overexpressing cell lines cause a decrease in survival. (B) shRNA of ligands causes an increase in overall survival. (C) Ligand overexpressing cell lines caused an increase in speed and size of tumor volume. (D) shRNA of ligands caused a decrease in speed of growth and tumor volume. (E) As expected, overexpression of ligands increased total tumor mass while shRNA of ligands decreased overall tumor mass.

Decreased Autocrine EGFR Signaling in Metastatic Breast Cancer Cells Inhibits Tumor Growth in Bone and Mammary Fat Pad

Nicole K. Nickerson¹, Khalid S. Mohammad^{2,3}, Jennifer L. Gilmore¹, Erin Crismore¹, Angela Bruzzaniti^{4,5}, Theresa A. Guise^{2,3} and John Foley^{1,3,5,6}.

Affiliations:

1. Medical Sciences Program, Indiana University School of Medicine, Bloomington, IN 47405
2. Division of Endocrinology, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN 46202
3. Indiana University Cancer Center, Indiana University School of Medicine, Indianapolis, IN 46202
4. Department of Oral Biology, Indiana University School of Dentistry, Indianapolis, IN 46202
5. Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN 46202
6. Department of Dermatology, Indiana University School of Medicine, Indianapolis, IN 46202

Keywords: Breast Cancer; Metastasis; EGFR; Amphiregulin; Bone

Corresponding Author: John Foley
Medical Sciences
Indiana University
Jordan Hall 104
Bloomington, IN 47405
Phone: 812-855-3189
E-mail: jgfoley@indiana.edu

Abstract

Breast cancer metastasis to bone triggers a vicious cycle of tumor growth linked to osteolysis. Breast cancer cells and osteoblasts express the epidermal growth factor receptor (EGFR) and produce ErbB family ligands, suggesting participation of these growth factors in autocrine and paracrine signaling within the bone microenvironment. EGFR ligand expression was profiled in the bone metastatic MDA-MB-231 cells (MDA-231), and agonist-induced signaling was examined in both breast cancer and osteoblast-like cells. Both paracrine and autocrine EGFR signaling were inhibited with a neutralizing amphiregulin antibody, PAR34, whereas shRNA to the EGFR was used to specifically block autocrine signaling in MDA-231 cells. The impact of these was evaluated with proliferation, migration and gene expression assays. Breast cancer metastasis to bone was modeled in female athymic nude mice with intratibial inoculation of MDA-231 cells, and cancer cell-bone marrow co-cultures. EGFR knockdown, but not PAR34 treatment, decreased osteoclasts formed *in vitro* ($p<0.01$), reduced osteolytic lesion tumor volume ($p<0.01$), increased survivorship *in vivo* ($p<0.001$), and resulted in decreased MDA-231 growth in the fat pad ($p<0.01$). Fat pad shEGFR-MDA-231 tumors produced in nude mice had increased necrotic areas and decreased CD31-positive vasculature. shEGFR-MDA-231 cells also produced decreased levels of the proangiogenic molecules macrophage colony stimulating factor-1 (MCSF-1) and matrix metalloproteinase 9 (MMP9), both of which were decreased by EGFR inhibitors in a panel of EGFR-positive breast cancer cells. Thus, inhibiting autocrine EGFR signaling in breast cancer cells may provide a means for reducing paracrine factor production that facilitates microenvironment support in the bone and mammary gland.

Keywords: Breast Cancer; Metastasis; EGFR; Amphiregulin; Bone

Introduction

The epidermal growth factor (EGFR) has long been recognized as a therapeutic target in breast and other epithelial cancers due to its ability to potently stimulate cell proliferation, motility, and invasion. The EGFR is activated by a family of ligands that include epidermal growth factor (EGF), Amphiregulin (AREG), transforming growth factor α (TGF α), heparin-binding EGF (HB-EGF), betacellulin, epiregulin, epigen, and Neuregulin 2 β [1]. These factors are synthesized as plasma membrane proteins tethered by a transmembrane domain, requiring proteolytic cleavage to be accessible to receptors [2]. These individual ligands may induce differential signaling pathways downstream of the EGFR, both from the plasma membrane and intracellular compartments, which can result in certain ligands being more efficient stimulators of proliferation [1,3,4,5,6]. Breast cancer cells frequently express the EGFR, one or more of its ligands and proteases that shed the ligands, resulting in autocrine signaling that may contribute to their rapid growth and invasive behavior.

The EGFR is frequently expressed in the basal subtype of breast cancer, which typically lack the expression of estrogen receptor α (ER α), progesterone receptor (PR) and Her2 receptor, accounting for only ~15-20% of the total disease [7,8,9]. However, 50-75% of basal breast cancers express EGFR and are more aggressive than similar tumors lacking the receptor [10,11]. Co-expression of the ADAM17 protease and the TGF α ligand in primary basal tumors has been associated with reduced survival [12]. These observations suggest that more aggressive basal-like breast cancers have the capacity to be stimulated by autocrine EGFR signaling, whereas the ligands produced by other subtypes of breast cancer (luminal, HER2 positive) may serve as paracrine signaling molecules [13].

Models of breast cancer metastasis to specific organs have provided evidence that EGFR ligands mediate paracrine signaling with cells of the tumor environment. Recent gene expression profiling of a bone-homing MDA-231 subline found that MMP-1 (matrix metalloproteinase 1) and ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin motifs) were upregulated, leading to increased AREG shedding [14]. The increased AREG appeared to signal via the EGFR present on osteoblasts, leading to reduced production of osteoprotegerin, the decoy ligand to the major controller of osteoclast differentiation and activation, receptor for nuclear factor $\kappa\beta$ ligand (RANKL) [14]. Increased osteoclast numbers and activity is a key element in the growth of breast cancer cells in the bone [15]. The metastatic growth of these MDA-231 sublines could be inhibited by the EGFR-targeted therapeutics cetuximab or gefitinib alone, or in combination with other targeted agents [14,16,17].

Autocrine activation of EGFR on breast cancer cells may also influence signaling with the bone microenvironment. Models of bone metastasis have provided evidence that cancer cell activation of EGFR often leads to the production of paracrine signaling molecules necessary for survival and rapid growth within the bone. Among the most well characterized factors that facilitate the growth of cancer cells in the bone is parathyroid hormone related protein (PTHrP), which signals through its receptor on osteoblasts, and leads to an increase of RANKL expression and increased osteoclast activity [18,19]. Autocrine activation of EGFR is a major regulator of PTHrP in both breast and lung cancers [20]. Intriguingly, the stimulation of the PTH receptor on osteoblasts stimulates the expression and shedding of AREG, thus potentially initiating a second autocrine loop of EGFR signaling in osteoblasts [21,22]. Taken together, autocrine EGFR-driven cytokine production, as well as paracrine interactions of the EGFR ligands themselves,

both appear to drive growth of bone-metastatic lesions suggesting various agents that disrupt this signaling could be effective treatments for breast cancer metastasis to bone.

In this study, we evaluated EGFR ligand expression by a bone-homing subline of the human breast cancer cell line MDA-231, with regard to their impact on specific malignant phenotypes and breast cancer cell signaling, as well as paracrine signaling to a mouse bone cell line. To specifically inhibit autocrine signaling in the MDA-231 cells we reduced EGFR expression by a lentiviral shRNA, and to inhibit both autocrine and paracrine EGFR signaling, an AREG neutralizing antibody was used. Finally, we evaluated the impact of altered autocrine and paracrine signaling on MDA-231 cell growth *in vitro* as well as *in vivo*, in the bone and mammary fat pad.

Materials and Methods

Ethics Statement

Animal care and experiments were approved by the Indiana University Animal Care and Use Committee (IACUC), OLAW assurance #94094-01, protocol #10-014.

Cell lines and cell culture

MDA-MB-231 cells were obtained from T. Guise [18] and MC3T3 cells were obtained from A. Robling [23], and were grown in DMEM (Sigma, St Louis, MO) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) and 10ng/mL insulin (Sigma). S1T3 and NS2T2A1 cells were both obtained from Z. Bouizar [24], and grown in a 50:50 mixture of RPMI and DMEM:F12 (Sigma) supplemented with 10% FBS. SUM149 cells were purchased from Asterand (Detroit, MI) and grown in F12 Hams (Sigma) supplemented with 10% FBS (Atlanta Biologicals).

Production of shEGFR-MDA-231 and shControl cells: MDA-MB-231 cells [18] were plated in 12-well dishes and grown to 50% confluence. 10 μ L of either EGFR shRNA lentiviral particles (Santa Cruz Biotechnology, Santa Cruz, CA) or Control shRNA lentiviral particles (Santa Cruz Biotechnology) with 6 μ g/mL polybrene (Santa Cruz Biotechnology) was added to the wells. Cells were grown for 24 hours before removal of lentiviral particles, then grown another 24 hours before 1.5 μ g/mL puromycin selection. Pooled colonies were tested for EGFR expression by western blotting, and cultures maintained in media with 1. 5 μ g/mL puromycin.

Animal injections and therapeutic dosing

For intratibial inoculation, either 7.5 x 10³ MDA-MB-231, shEGFR-MDA-231, or shControl cells were inoculated into the left tibia of 3-4 week old female athymic nude mice (Harlan, Indianapolis, IN). Mice were anesthetized with 5% isoflurane and laid in a dorsal

position. Autoclaved 100 μ L Hamilton syringes with 27 gauge needles were used to puncture the skin at the proximal end of the left tibia. The syringe was gently pushed through the epiphysis to about 3mm deep to assure it passed through metaphysis, and 10 μ L of cell suspension was inoculated slowly over 20 seconds. Mice were anesthetized with 5% Isoflurane, and lay in a prone position for weekly radiography to monitor lesion progression (35kV for 10 seconds; Faxitron, Lincolnshire, IL). Animals were x-rayed at 2x magnification, and lesions were first detected at 14 days post inoculation. End point for these studies was 4-6 weeks after tumor inoculation, or earlier if the size of the x-ray lesion reached 25% of the upper tibia area, swelling of knee region exceeded 2-fold the diameter of the non-injected limb, the limb could not be used for ambulation, or the animals displayed signs of excessive pain, per our veterinarian-guided animal protocol and pain-scale. Osteolytic area on x-ray was measured using ImageJ software (NIH).

Upon sacrifice, hind limbs were removed and fixed in 10% neutral buffered formalin for 48 hours, and then 70% EtOH for at least 24 hours. After microCt imaging, bones were decalcified (10% EDTA for 1 week), and embedded in paraffin. Tibiae were sectioned at 7 μ M in the sagittal plane and mid-sagittal sections were stained with hematoxylin and eosin (H&E) or tartrate resistant acid phosphatase (TRAP). TRAP staining for osteoclasts was performed using an azo-dye coupling method with fast red violet LB salt (F-3881, Sigma) as described [25]. After rehydration through graded alcohols, sections were incubated in freshly prepared TRAP stain at 37°C for 15 minutes, counterstained in hematoxylin, and mounted in glycerin jelly. Serial slides were stained with hematoxylin and eosin (H&E) as described [26]. For this study, PAR34 antibody was administered for 4 weeks through intraperitoneal injection once weekly at 10mg/kg in a sterile 0.9% saline solution [27].

For mammary fat pad tumors, shControl or shEGFR-MDA-231 cells were combined with 50% Matrigel and inoculated at 1×10^6 cells in 100 μ l total volume in the first mammary fat pad of female athymic nude mice, aged 3-4 weeks (Harlan). One group of shControl mice was administered PAR34 at 10mg/kg/week by intraperitoneal injection. Tumors were measured twice weekly for length (L) and width (W), and tumor volume (V) calculated as: $V = (L \times W^2) \times 0.5$.

Micro-CT

Fixed tibiae were scanned using a SkyScan micro-CT (SkyScan 1172; SkyScan, Belgium) as previously described [28], with the following scanner settings: voltage, 60kV; resolution, 6 μ m; 0.5mm aluminum filter; stage rotation, 0.7; and frame-averaging, 2. Flat-field corrections were used to minimize background noise. NRecon software (SkyScan), was used to reconstruct the images, with post-alignment optimization performed for each separate tibia. CTan software (SkyScan), was used to analyze reconstructed images, separating bone from surrounding soft tissue with a threshold range of 100 to 255 (binarized 0-255 scale). Bone volume was reported from analysis of 700 sections per tibia. 3D images were obtained using MeshLab software (MeshLab, 3D-CoForm) with smoothing option.

Trabecular bone analysis regions were chosen in the secondary spongiosum, with a consistent total length of 1mm measured for each tibia. Region of interest was chosen as only the internal bone cavity containing trabecular bone with cortical bone excluded.

Osteoclastogenesis assays

Osteoclastogenesis assays were performed as in [29]. Briefly, 1-4 month old mice were euthanized, hind limbs dipped in 70% ethanol and removed at hip. Femur and tibia ends were cut to expose the bone marrow cavity, and each marrow cavity flushed with 5-10mL of DMEM cell culture medium. 50 μ L of cell suspension was mixed with 450 μ L of 2% acetic acid to lyse

red blood cells, and remaining cells counted. 4×10^5 cells were plated in each well of a 24-well dish with 60ng/mL RANKL (PeproTech) for 3 days. After 3 days, 2×10^3 MDA-231, shControl, or shEGFR-MDA-231 cells were plated with the bone marrow cells, with 60ng/mL RANKL and 10ng/mL MCSF (PeproTech) and grown for 3 days before TRAP staining. For TRAP staining, cells were washed with 1xPBS, fixed with ice cold methanol for 10 minutes, and stained in fresh TRAP solution for 15 minutes at 37°C. TRAP solution was replaced with 1xPBS for cell counting under the microscope.

Statistical analysis

Results of *in vitro* experiments are expressed as the mean \pm SD of triplicate or quadruplicate measures of independent replicates for single experiments. Results of *in vivo* experiments are expressed as the mean \pm SEM of three to six replicates of samples taken from ten individual animals. All statistical comparisons were based on two-tailed analysis of the Student's *t* test. A *P* value of <0.05 was considered to be significant.

Results

Amphiregulin is highly secreted by MDA-231 cells

Previously we determined a subline of the aggressive breast cancer cell line MDA-231 efficiently colonizes mouse bone after intracardiac inoculation, expresses high levels of EGFR protein and modest levels of the ErbB2 and ErbB3 receptors, and sheds AREG [20]. To more completely evaluate EGFR ligand production in these cells, we examined the expression of five EGFR ligands, including epidermal growth factor (EGF), AREG, betacellulin, heparin-binding EGF (HB-EGF), and transforming growth factor α (TGF α) using ELISA for both conditioned media and membrane extracts. MDA-231 cells release high levels of AREG (0.048pM), and maintain similar concentrations associated with the membrane fraction. Surprisingly, higher levels of HB-EGF also remained associated with the cell membrane (0.254pM), with lower levels (0.008pM) detectable in the media (Fig 1A). Low concentrations of TGF α were present on the membrane fraction (0.008pM) while higher levels were detected in the media (0.063pM). Betacellulin was present in low concentrations (0.004pM) and EGF protein was undetectable using this methodology (Fig 1A). In terms of autocrine signaling *in vitro*, AREG appears to be shed at the highest concentrations, while high levels of membrane-associated HB-EGF indicate that this could be the predominant ligand if it were cleaved from the membrane.

Amphiregulin activates EGFR phosphorylation on both MDA-231 and MC3T3 cells

To determine if the impact of EGFR signaling in the bone microenvironment is similar to that of breast cancer cells, we used a mouse preosteoblastic cell line MC3T3 as a model to compare receptor phosphorylation induced by exogenous ligand treatment. Here, we used 100 nM recombinant human ligands (AREG, TGF α , and HB-EGF), as well as recombinant human EGF (10 nM) to serve as the prototype ligand. MC3T3 or MDA-231 cells were treated with EGF,

AREG, TGF α , or HB-EGF, followed by western blotting with their respective phospho-specific antibodies. Modest levels of basal EGFR tyrosine phosphorylation could be detected in MDA-231 cells at Y992 and Y1086, whereas baseline EGFR levels could not be detected in the MC3T3 line with any of the phospho-specific antibodies (Fig 1B). Human EGF and HB-EGF were able to induce receptor phosphorylation on both MDA-231 and MC3T3 cells, as detected with 4G10, a pan phosphotyrosine antibody, as well as the other site-specific antibodies. Exogenous AREG induced modest phosphorylation of some residues in MDA-231 cells compared to EGF, but appeared to increase phosphorylation of all tested residues in MC3T3 cells. We noted that TGF α caused very little phosphorylation in the human cells and was not able to induce detectable changes in EGFR phosphorylation in mouse MC3T3 cells. Though both AREG and TGF α are shed and capable of inducing receptor phosphorylation in MDA-231 cells, AREG appears to be the highest cleaved ligand and it is able to potently activate the EGFR on mouse osteoblast-like MC3T3 cells providing the rationale to target this ligand as the main inducer of both autocrine breast cancer signaling and paracrine receptor signaling in mouse tissues.

shRNA to the EGFR causes a decrease in migration and PTHrP expression in MDA-231 cells

To inhibit breast cancer cell autocrine and paracrine signaling, we used shRNA to the EGFR as well as a monoclonal antibody (PAR34) (Figure S1). To reduce autocrine EGFR signaling in the MDA-231 line, cells were transduced with a lentiviral shRNA to the receptor (shEGFR-MDA-231 cells) or a shRNA scrambled control (shControl). As detected by western blot, there was a 64% knockdown of the EGFR as compared to MDA-231 or shControl cells, and this knockdown did not affect levels of other EGFR family receptors (Fig 2A). Introduction of the shEGFR construct had no effect on production of AREG, TGF α , or HB-EGF mRNA production

(data not shown). We verified by ELISA that ligand protein levels were not disrupted by the shEGFR construct, as AREG, TGF α , and HB-EGF were present in the media or on cell membranes at the same levels in shEGFR-MDA-231 cells, as compared to MDA-231 and shControl cells (Fig 2B). Treatment of MDA-231 or shControl cells with PAR34 antibody or control IgG had no effect on ligand expression of AREG, TGF α , or HB-EGF (Fig 2B). As expected, a decrease ($p<0.05$) in PTHrP levels in the shEGFR-MDA-231 cells was observed as compared to control cells (Fig 2C), indicative of reduced autocrine EGFR signaling.

We then examined impact of PAR34 on breast cancer cells grown *in vitro*. PAR34 inhibited exogenous AREG-induced phosphorylation of tyrosines 992 and 1173 in MDA-231 cells, when compared to IgG control (Figure S2B), and this inhibition was AREG-specific, as PAR34 did not inhibit stimulation by EGF. Similar inhibition of exogenous phosphorylation was noted in the non-tumorigenic, epithelial breast cell line S1T3 (S1 cells).

To further test the impact of autocrine EGFR signaling inhibition by PAR34 antibody and shRNA knockdown, cell proliferation and migration were examined *in vitro*. EGFR signaling has been reported to stimulate motility, but does not induce proliferation in MDA-231 cells [30,31]. Using the MTT assay we found that shEGFR-MDA-231 cells and controls treated with PAR34 proliferated at a similar rate to non-treated controls (Fig 2C). As shown in Figure 2D, PAR34 inhibited migration ($p<0.001$) of both MDA-231 and shControl cells by 20%, and migration was decreased ($p<0.001$) by 65% in shEGFR-MDA-231 cells relative to controls. Taken together, these *in vitro* assays confirm that inhibition of EGFR by PAR34 or shRNA decreases breast cancer cell motility.

PAR34 treatment modifies the trabecular patterning factor of bone

To examine the impact of inhibiting AREG signaling within the bone, we first evaluated PAR34 antibody treatment in non-tumor bearing animals. Female athymic nude mice aged 3-4 weeks received intraperitoneal injections of PAR34, at 10mg/kg/week, for 4 weeks. Upon sacrifice, tibiae were removed and prepared for microCT and histological sectioning. While PAR34 treatment did not affect the gross bone structure, as analyzed by both x-ray and microCT (data not shown), microCT showed a decrease ($p<0.001$) in trabecular pattern factor in PAR34 tibiae when compared to vehicle treated animals (Table S1 and Figure S2A). We also evaluated osteoclasts present in the newly deposited bone under the hypertrophic zone of growth plate chondrocytes, and observed an increase ($p<0.01$) in the number of these cells per bone surface area in PAR34 treated animals versus control animals (Figure S2B and Table S1). Overall, PAR34 treatment influenced bone growth, thus validating this dose and schedule as effective for targeting bone *in vivo*.

shEGFR-MDA-231 cells produce smaller tumors in bone

To examine global inhibition of AREG signaling, or to specifically reduce cancer cell EGFR signaling during osteolytic lesion growth within the bone, female athymic nude mice (aged 3-4 weeks) were inoculated in the left tibia with MDA-231, shControl, or shEGFR-MDA-231 cells. Intratibial inoculation was chosen to insure that differential motility of the shEGFR-MDA-231 did not inhibit colonization of the bone. Three days after inoculation, treatment of one group of MDA-231 inoculated mice was initiated with weekly intraperitoneal injection of PAR-34 antibody (10mg/kg). The MDA-231, shControl, and PAR34 treated groups had extensive osteolytic lesion destruction as detected by x-ray and microCT at the experimental end-point, while the majority of shEGFR-MDA-231 mice had smaller regions of distinct bone loss measured by x-ray (Fig 3A). All PAR34 treated animals required sacrifice after the 3-week time

point, as they displayed experimental end-point criterion including maximum x-ray lesion size, swelling of the injected limb, or ambulation difficulties. Survival was increased ($p<0.001$) in shEGFR-MDA-231 tumor-bearing mice as compared to those inoculated with MDA-231 or shControl (Fig 3B), and osteolytic lesion size was decreased ($p<0.01$) in shEGFR-MDA-231 animals (Fig 3C). Although large lesions were readily apparent in the reconstruction of microCT scans from the MDA-231, shControl, or PAR34 groups, total tibia head bone volume was not significantly different as compared to the shEGFR-MDA-231 group (Fig 3D).

Examination of H&E stained tibiae from all groups verified large, destructive tumors within the MDA-231, PAR34 treated, and shControl groups (Materials and Methods S1). Interestingly, shEGFR-MDA-231 animals had smaller tumors ($p<0.01$) that remained within the bone marrow cavity (Fig 4A). Surprisingly, the PAR34 treated animals had a larger tumor volume ($p<0.05$) when compared to controls (Fig 4B). Tartrate resistant acid phosphatase (TRAP) staining indicated the number of osteoclasts per tumor bone interface in shEGFR-MDA-231 bones trended toward a decrease in comparison to MDA-231 or shControl tumor-bearing tibiae (Fig 4C). Additionally, we observed an increase in osteoclasts per tumor bone interface, though this was not significant (Fig 4C).

Thus, it appears that decreased EGFR was sufficient to reduce the size of osteolytic lesions and tumor volume within bone. Conversely, PAR34 antibody enhanced MDA-231 growth within the bone.

Modulation of EGFR signaling impacts osteoclastogenesis in vitro

We also examined the effects of EGFR knockdown or PAR34 treatment using an *in vitro* osteoclastogenesis assay, whereby MDA-231 or shEGFR-MDA-231 cells were co-cultured with mouse bone marrow (BM) to determine if osteoclast formation could be increased. As seen in

Figure 5A, co-culture of BM with shEGFR-MDA-231 cells stimulated fewer ($p<0.01$) osteoclasts than control cell co-cultures, correlating with the decreased osteolytic lesion size *in vivo*.

Next we evaluated the impact of PAR-34 and exogenous AREG on various permutations of the co-culture assay. We also observed that PAR34 antibody caused an increase in osteoclasts in BM alone (Fig 5B, $p<0.01$) or co-cultures with MDA-231 ($p<0.01$) and the shEGFR-MDA-231 cells ($p<0.001$) (Fig 5C&5D). In contrast, exogenous AREG ligand failed to increase osteoclasts in BM, but stimulated the formation in co-cultures that contained MDA-231 cells (Fig 5C, $p<0.01$). Intriguingly exogenous ligand did not increase osteoclast number in the shEGFR-MDA-231 containing co-cultures (Fig 5D). The impact of PAR-34 on BM alone or cancer cell co-cultures generally corresponded with *in vivo* findings where the antibody treatment produced increased osteoclasts in non-tumor bearing bones and increased tumor size in cancer cell injected bones.

To further investigate the impact of EGFR signaling inhibitors on osteoclastogenesis, BM and MDA-231 co-cultures were treated with a range of concentrations of gefitinib, a small molecule EGFR inhibitor [32]. As shown in Figure S3, 1 μ M gefitinib also increased osteoclasts ($p<0.001$) in co-cultures, but showed a trend toward decreased formation in BM cultures alone. These findings coupled with those from PAR-34 treatments suggest that different EGFR inhibitors can have distinct impacts on osteoclastogenesis and in some cases they may enhance it.

shEGFR-MDA-231 cells produce smaller mammary fat pad tumors

To determine if the reduced growth of the shEGFR-MDA-231 cells in bone was specific to that microenvironment, we examined the *in vivo* growth rate of mammary fat pad tumors

produced by shControl or shEGFR-MDA-231 cells. A group of shControl-inoculated animals were treated with weekly intraperitoneal injections of PAR34 (10mg/kg). As shown in Figure 6, tumor volume measures and final masses were decreased ($p<0.01$) in shEGFR-MDA-231 tumors as compared to shControl (Fig 6A&6B). While PAR34 treatment trended towards reduced tumor volume and mass, these results were not significant compared to shControl tumors (Fig 6A&6B). Histological analysis revealed an increased ($p<0.05$) necrotic area in shEGFR-MDA-231 tumors, despite unchanged cell proliferation as detected by an anti-Ki67 antibody (Table 1). However, fewer vessels ($p<0.001$) were stained by anti-CD31 antibody in the shEGFR-MDA-231 tumors than shControl cells (Table 1 and Fig 6C). Thus, reduced growth *in vivo* of the shEGFR-MDA-231 cells was observed in the mammary fat pad, likely correlated with reduced vascularization of the tumor.

Decreased EGFR signaling causes a reduction in proangiogenic factor expression

To explore the molecular basis of the reduced vasculature of the mammary fat pad tumors produced by shEGFR-MDA-231 cells, we first examined changes in expression of vascular endothelial growth factor (VEGF). Previous work has reported VEGF is regulated in breast cancer cells by EGFR signaling [33], however we observed no differences in control versus shEGFR-MDA-231 cells (Fig 7A). Previous publications have noted that EGFR signaling regulates macrophage colony-stimulating factor-1 (MCSF-1) expression in murine cancer cells [34]. MCSF-1 could influence angiogenesis by recruiting macrophages or various progenitors from the bone marrow, which could produce VEGF or directly contribute to neoangiogenesis [35,36]. MCSF-1 levels were lower ($p<0.05$) in shEGFR-MDA-231 cells when compared to controls, as measured by ELISA (Fig 7B).

To verify this finding is due to EGFR inhibition and not off target effects of the shRNA construct, we examined MCSF-1 levels in a panel of breast cancer cell lines following treatment with the small molecule EGFR inhibitor PD153035 or PAR34. PD153035 reduced MCSF-1 secretion from the parental MDA-231 ($p<0.01$), shControl ($p<0.01$), SUM149 ($p<0.05$), and the tumorigenic epithelial breast cancer cell line NS2T2A1 ($p<0.01$) (Fig 7C). PAR34 decreased MCSF-1 levels in MDA-231, shControl and NS2TA1 cells ($p<0.05$) in which AREG is the predominant ligand, but not in SUM149 cells. We further evaluated the shEGFR-MDA-231 and control cells for matrix metalloproteinase 9 (MMP-9), a protease that promotes angiogenesis by releasing VEGF that is bound to extracellular matrix [37]. As shown in Figure 7D, MMP-9 levels were markedly reduced in cell extracts of shEGFR-MDA-231, as compared to shControl and MDA-231. Also, PAR34 reduced expression of the protease in SUM149 and NS2TA1 cell lines. PD153035 inhibition (6-hrs) had no effect on MMP-9 levels. These findings suggest that autocrine EGFR signaling regulates at least two proangiogenic factors in breast cancer cell lines, and disruption of receptor signaling would be predicted to reduce vascularization and decreased growth of tumors.

Discussion

In this study, we found that reduced EGFR expression decreased MDA-231 cell growth within bone and the mammary gland. Previous studies suggested that EGFR signaling promotes growth *in vivo* as part of paracrine relationships between breast epithelia-derived cells and the microenvironment. The mammary epithelium expresses both EGFR and its ligands EGF, TGF α , and AREG, suggesting a potential for autocrine signaling [38,39]; however, elegant recombination experiments established that mammary gland ductal outgrowth requires EGFR expression in the fat pad, and AREG expression in the epithelium [39,40,41]. In lung and brain metastasis models, epigen or HB-EGF expressed by MDA-231 cells signal to the EGFR on endothelial cells to facilitate colonization of these organs. In a model of bone metastasis, breast cancer cell derived AREG is thought to signal to the osteoblast, facilitating osteoclast formation and driving osteolytic destruction. In contrast, autocrine EGFR signaling is typically associated with proliferation of epithelial cancers [13]. Since the MDA-231 line bears a mutation in K-ras, which activates the MAPK cascade the major driver of mitogenesis downstream of the EGFR [30,31], this line represented an ideal system for modulating receptor levels without reducing cell proliferation. The shEGFR-MDA-231 cells did not exhibit alterations in the expression of ligands or other ErbB receptors, and had identical rates of proliferation as compared to control lines (Fig. 2). Decreased EGFR expression in the MDA-231 resulted in slower tumor growth in both the bone and the mammary gland (Figs. 3 and 6). The shEGFR-MDA-231 cells produced smaller osteolytic lesions *in vivo* and induced the formation of fewer osteoclasts *in vitro* relative to controls (Figs. 3 and 5). Consistent with a central role for the breast cancer cell EGFR in the

stimulation of osteoclastogenesis, the addition of exogenous AREG to co-cultures containing shEGFR-MDA-231cell failed to induce increased numbers of the bone resorbing cells (Fig. 5). We conclude that autocrine EGFR signaling contributes to MDA-231 tumor growth in bone and the mammary gland independent of driving cancer cell proliferation.

There is a growing appreciation that EGFR signaling in epithelial cancer cells stimulates the expression of many chemokines, cytokines, growth factors, and receptors that facilitate paracrine interactions with non-cancer cells of the tumor microenvironment [20,42,43,44]. EGFR signaling controls the expression of VEGF isoforms, which are pivotal factors that control angiogenesis in many tumors [33,42,45]. We did not detect differences in VEGF levels in the shEGFR-MDA-231 cells, consistent with previous studies of MDA-231 sublines [46]. However, we did detect decreases in proangiogenic factors such as MCSF-1 and MMP-9 in the shEGFR-MDA-231 cells, as well as a panel of breast cancer cell lines treated with an EGFR tyrosine kinase inhibitor or PAR-34. Reduced expression of MCSF-1 and MMP-9 would likely influence the growth of the MDA-231 cells in both the mammary fat pad and the bone. Also, the decreased production of PTHrP would be expected to contribute to reduced growth of the MDA-231 line in bone. Previously, it has been shown that PTHrP antibody inhibition dramatically decreased the number of osteoclasts per tumor bone interface, coupled with decreased osteolytic lesion size [18]. Consistent with the reduction of PTHrP, we observed a trend in reduction of osteoclasts in shEGFR-MDA-231 inoculated tibiae; also, *in vitro* studies indicated that shEGFR-MDA-231 cells generate fewer osteoclasts than control MDA-231 cells (Fig. 5A). It is likely that the EGFR on breast cancer cells controls the expression of many additional cytokines and growth factors that mediate tumor cell-microenvironment interactions, both in primary tumors and sites of metastasis.

Our attempt to block both autocrine and paracrine EGFR signaling by antagonizing AREG interaction with its receptor, using PAR34 antibody, produced surprising results. Given that we had previously found that AREG was the major ligand controlling PTHrP expression in MDA-231 cells [20], it was not surprising that PAR-34 decreased MCSF-1 and MMP-9 production. However, the antibody only modestly inhibited MDA-231 cell motility in comparison to knockdown of the receptor (Fig. 2D). This raises questions as to whether the various ligands might exhibit differential impacts on cell motility and growth factor production, as previously established for some of these agonists in the stimulation of EGFR-dependent cellular proliferation [3]. Also, the failure of the antibody to potently inhibit motility may reflect its inability of block signaling of the EGFR from internal compartments such as the endosome [47]. *In vivo*, we observed that PAR-34 treatment increased MDA-231 tumor growth within the bone, while also increasing active osteoclasts at the tumor bone interface. Correspondingly, PAR34 increased osteoclastogenesis in BM alone as well as BM cancer cell co-cultures, and increased osteoclast numbers below the growth plate of non-tumor bearing. These later findings suggest that PAR-34 may induce a higher baseline bone turnover, and this could contribute to the increased tumor growth that we observed in our *in vivo* experiments. Although an impact on baseline osteoclastogenesis of BM cultures was not observed with gefitinib, we found that this EGFR inhibitor also increased osteoclastogenesis MDA-231 containing co-cultures. Together these unanticipated findings lead us to speculate that AREG-EGFR signaling in the bone marrow microenvironment may influence other processes besides osteoblast differentiation and subsequent osteoclastogenesis. Recent reports indicate that EGFR signaling decreases hematopoietic stem cell mobilization in response to G-CSF [48]. Derivatives of hematopoietic stem cells include osteoclasts, monocytes, myeloid suppressor cells and megakaryocytes that all

could influence the growth of breast cancer cells in the bone [49]. Our unexpected findings with AREG antibody treatments of cancer cells in the bone marrow encourage a more careful analysis of the impact of various inhibitors on EGFR signaling on all cell types in the breast cancer bone metastasis microenvironment.

In conclusion, EGFR knockdown in MDA-231 cells reduced their motility and production of secreted factors that stimulate osteolytic lesion growth and angiogenesis *in vitro*. *In vivo*, EGFR knockdown in MDA-231 cells reduced tumor growth both in the mammary fat pad and the bone. MDA-231 cells act as a model for triple negative breast cancers, so these findings raise the possibility that interventions that could reduce EGFR expression in triple-negative breast cancer cells might provide therapeutic benefit to patients with metastatic disease.

Acknowledgements

We thank Dr. Alex Robling for the gift of MC3T3 cells and Dr. Matthew Allen for his help with the SkyScan software. We thank Drs. Kenneth Nephew and Curt Balch for their critical review of the manuscript. We are grateful to the Indiana University Advanced Visualization Lab, an affiliate of the Pervasive Technology Institute, for their help with 3D image reconstruction.

References

1. Wilson KJ, Gilmore JL, Foley J, Lemmon MA, Riese DJ, 2nd (2009) Functional selectivity of EGF family peptide growth factors: implications for cancer. *Pharmacol Ther* 122: 1-8.
2. Gschwind A, Hart S, Fischer OM, Ullrich A (2003) TACE cleavage of proamphiregulin regulates GPCR-induced proliferation and motility of cancer cells. *Embo J* 22: 2411-2421.
3. Kochupurakkal BS, Harari D, Di-Segni A, Maik-Rachline G, Lyass L, et al. (2005) Epigen, the last ligand of ErbB receptors, reveals intricate relationships between affinity and mitogenicity. *J Biol Chem* 280: 8503-8512.
4. Roepstorff K, Grandal MV, Henriksen L, Knudsen SL, Lerdrup M, et al. (2009) Differential effects of EGFR ligands on endocytic sorting of the receptor. *Traffic* 10: 1115-1127.
5. Willmarth NE, Baillo A, Dziubinski ML, Wilson K, Riese DJ, 2nd, et al. (2009) Altered EGFR localization and degradation in human breast cancer cells with an amphiregulin/EGFR autocrine loop. *Cell Signal* 21: 212-219.
6. Willmarth NE, Ethier SP (2006) Autocrine and juxtacrine effects of amphiregulin on the proliferative, invasive, and migratory properties of normal and neoplastic human mammary epithelial cells. *J Biol Chem* 281: 37728-37737.
7. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, et al. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 98: 10869-10874.
8. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, et al. (2002) Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 415: 530-536.
9. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, et al. (2002) A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 347: 1999-2009.
10. Yang XR, Chang-Claude J, Goode EL, Couch FJ, Nevanlinna H, et al. Associations of breast cancer risk factors with tumor subtypes: a pooled analysis from the Breast Cancer Association Consortium studies. *J Natl Cancer Inst* 103: 250-263.
11. Voduc KD, Cheang MC, Tyldesley S, Gelmon K, Nielsen TO, et al. Breast cancer subtypes and the risk of local and regional relapse. *J Clin Oncol* 28: 1684-1691.
12. Kenny PA, Bissell MJ (2007) Targeting TACE-dependent EGFR ligand shedding in breast cancer. *J Clin Invest* 117: 337-345.
13. Foley J, Nickerson NK, Nam S, Allen KT, Gilmore JL, et al. EGFR signaling in breast cancer: Bad to the bone. *Semin Cell Dev Biol*.
14. Lu X, Wang Q, Hu G, Van Poznak C, Fleisher M, et al. (2009) ADAMTS1 and MMP1 proteolytically engage EGF-like ligands in an osteolytic signaling cascade for bone metastasis. *Genes Dev*.
15. Guise TA, Mohammad KS, Clines G, Stebbins EG, Wong DH, et al. (2006) Basic mechanisms responsible for osteolytic and osteoblastic bone metastases. *Clin Cancer Res* 12: 6213s-6216s.
16. Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, et al. (2009) Genes that mediate breast cancer metastasis to the brain. *Nature*.

17. Gupta GP, Nguyen DX, Chiang AC, Bos PD, Kim JY, et al. (2007) Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. *Nature* 446: 765-770.
18. Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, et al. (1996) Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J Clin Invest* 98: 1544-1549.
19. Thomas RJ, Guise TA, Yin JJ, Elliott J, Horwood NJ, et al. (1999) Breast cancer cells interact with osteoblasts to support osteoclast formation. *Endocrinology* 140: 4451-4458.
20. Gilmore JL, Scott JA, Bouizar Z, Robling A, Pitfield SE, et al. (2008) Amphiregulin-EGFR signaling regulates PTHrP gene expression in breast cancer cells. *Breast Cancer Res Treat* 110: 493-505.
21. Qin L, Partridge NC (2005) Stimulation of amphiregulin expression in osteoblastic cells by parathyroid hormone requires the protein kinase A and cAMP response element-binding protein signaling pathway. *J Cell Biochem* 96: 632-640.
22. Qin L, Qiu P, Wang L, Li X, Swarthout JT, et al. (2003) Gene expression profiles and transcription factors involved in parathyroid hormone signaling in osteoblasts revealed by microarray and bioinformatics. *J Biol Chem* 278: 19723-19731.
23. Yang J, Shah R, Robling AG, Templeton E, Yang H, et al. (2008) HMGB1 is a bone-active cytokine. *J Cell Physiol* 214: 730-739.
24. Berthon P, Goubin G, Dutrillaux B, Degeorges A, Faille A, et al. (1992) Single-steep transformation of human breast epithelial cells by SV40 large T oncogene. *Int J Cancer* 52: 92-97.
25. Gerstenfeld LC, Alkhiary YM, Krall EA, Nicholls FH, Stapleton SN, et al. (2006) Three-dimensional reconstruction of fracture callus morphogenesis. *J Histochem Cytochem* 54: 1215-1228.
26. Mohammad KS, Chirgwin JM, Guise TA (2008) Assessing new bone formation in neonatal calvarial organ cultures. *Methods Mol Biol* 455: 37-50.
27. Bhagavathula N, Nerusu KC, Fisher GJ, Liu G, Thakur AB, et al. (2005) Amphiregulin and epidermal hyperplasia: amphiregulin is required to maintain the psoriatic phenotype of human skin grafts on severe combined immunodeficient mice. *Am J Pathol* 166: 1009-1016.
28. Allen MR, Ruggiero SL (2009) Higher bone matrix density exists in only a subset of patients with bisphosphonate-related osteonecrosis of the jaw. *J Oral Maxillofac Surg* 67: 1373-1377.
29. Bruzzaniti A, Neff L, Sandoval A, Du L, Horne WC, et al. (2009) Dynamin reduces Pyk2 Y402 phosphorylation and SRC binding in osteoclasts. *Mol Cell Biol* 29: 3644-3656.
30. Davidson NE, Gelmann EP, Lippman ME, Dickson RB (1987) Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. *Mol Endocrinol* 1: 216-223.
31. Price JT, Tiganis T, Agarwal A, Djakiew D, Thompson EW (1999) Epidermal growth factor promotes MDA-MB-231 breast cancer cell migration through a phosphatidylinositol 3'-kinase and phospholipase C-dependent mechanism. *Cancer Res* 59: 5475-5478.
32. Moulder SL, Yakes FM, Muthuswamy SK, Bianco R, Simpson JF, et al. (2001) Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells in vitro and in vivo. *Cancer Res* 61: 8887-8895.

33. Ciardiello F, Caputo R, Bianco R, Damiano V, Fontanini G, et al. (2001) Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin Cancer Res* 7: 1459-1465.
34. Goswami S, Sahai E, Wyckoff JB, Cammer M, Cox D, et al. (2005) Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. *Cancer Res* 65: 5278-5283.
35. Roda JM, Sumner LA, Evans R, Phillips GS, Marsh CB, et al. Hypoxia-Inducible Factor-2{alpha} Regulates GM-CSF-Derived Soluble Vascular Endothelial Growth Factor Receptor 1 Production from Macrophages and Inhibits Tumor Growth and Angiogenesis. *J Immunol* 187: 1970-1976.
36. Voloshin T, Gingis-Velitski S, Bril R, Benayoun L, Munster M, et al. G-CSF supplementation with chemotherapy can promote revascularization and subsequent tumor regrowth: prevention by a CXCR4 antagonist. *Blood*.
37. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, et al. (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol* 2: 737-744.
38. Hens JR, Wysolmerski JJ (2005) Key stages of mammary gland development: molecular mechanisms involved in the formation of the embryonic mammary gland. *Breast Cancer Res* 7: 220-224.
39. Luetteke NC, Qiu TH, Fenton SE, Troyer KL, Riedel RF, et al. (1999) Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development* 126: 2739-2750.
40. Ciarloni L, Mallepell S, Brisken C (2007) Amphiregulin is an essential mediator of estrogen receptor alpha function in mammary gland development. *Proc Natl Acad Sci U S A* 104: 5455-5460.
41. Sebastian J, Richards RG, Walker MP, Wiesen JF, Werb Z, et al. (1998) Activation and function of the epidermal growth factor receptor and erbB-2 during mammary gland morphogenesis. *Cell Growth Differ* 9: 777-785.
42. Furugaki K, Moriya Y, Iwai T, Yorozu K, Yanagisawa M, et al. Erlotinib inhibits osteolytic bone invasion of human non-small-cell lung cancer cell line NCI-H292. *Clin Exp Metastasis*.
43. Gilmore JL, Gonterman RM, Menon K, Lorch G, Riese DJ, 2nd, et al. (2009) Reconstitution of amphiregulin-epidermal growth factor receptor signaling in lung squamous cell carcinomas activates PTHrP gene expression and contributes to cancer-mediated diseases of the bone. *Mol Cancer Res* 7: 1714-1728.
44. Lorch G, Gilmore JL, Koltz PF, Gonterman RM, Laughner R, et al. (2007) Inhibition of epidermal growth factor receptor signalling reduces hypercalcaemia induced by human lung squamous-cell carcinoma in athymic mice. *Br J Cancer* 97: 183-193.
45. Lee JE, Chung KW, Han W, Kim SW, Shin HJ, et al. (2004) Effect of estrogen, tamoxifen and epidermal growth factor on the transcriptional regulation of vascular endothelial growth factor in breast cancer cells. *Anticancer Res* 24: 3961-3964.
46. Scandlyn MJ, Stuart EC, Somers-Edgar TJ, Menzies AR, Rosengren RJ (2008) A new role for tamoxifen in oestrogen receptor-negative breast cancer when it is combined with epigallocatechin gallate. *Br J Cancer* 99: 1056-1063.
47. Balbis A, Posner BI Compartmentalization of EGFR in cellular membranes: role of membrane rafts. *J Cell Biochem* 109: 1103-1108.

48. Ryan MA, Nattamai KJ, Xing E, Schleimer D, Daria D, et al. Pharmacological inhibition of EGFR signaling enhances G-CSF-induced hematopoietic stem cell mobilization. *Nat Med* 16: 1141-1146.
49. Park SI, Soki FN, McCauley LK Roles of Bone Marrow Cells in Skeletal Metastases: No Longer Bystanders. *Cancer Microenviron*.

Figure Legends

Figure 1. *EGFR ligand expression and shedding in MDA-231 cells.* (A) ELISA measurement of media or membrane extracts from MDA-231 cells. Measurements were taken from two independent cultures and performed in triplicate. (B) Western blots of anti-EGFR and anti-phosphorylated tyrosine resides in MDA-231 or MC3T3 cells treated with EGF, AREG, TGF α , or HB-EGF.

Figure 2. *Characterization of the shEGFR-MDA-231 cell line.* (A) Extracts from MDA-231, shControl, and shEGFR-MDA-231 cells, probed with anti-EGFR or anti-ErbB2, ErbB3, or ErbB4 antibodies and anti- β -Tubulin (loading control). Histogram notes relative pixel density of EGFR protein of shEGFR-MDA-231 cells versus shControl and MDA-231 cells. (B) AREG, TGF α , and HB-EGF ELISA measurements of MDA-231, shControl, and shEGFR-MDA-231 cells, to verify no changes in basal or PAR34 treated ligand expression. ELISA measurements were performed in triplicate from two separate cultures. (C) Relative PTHrP mRNA levels in the shControl and shEGFR-MDA-231 cell lines. PTHrP was measured by qRT-PCR analysis and relative ratios of PTHrP mRNA to GAPDH mRNA levels were shown (mean of triplicate measures from a single experiment; bars, SD). (D) MTT proliferation assays were performed on shEGFR-MDA-231, MDA-231, and shControl cells, as well as PAR34-treated MDA-231 or shControl cells. MTT measurements were performed in quadruplicate, p<0.05. (E) 24 hour migration assay of shEGFR-MDA-231, MDA-231, and shControl cells, with PAR34 treatment to the latter two lines, p<0.001. Migrated cells were obtained from two separate migration wells, with four random fields chosen for counts from each well.

Figure 3. *In vivo analysis of autocrine or paracrine inhibition of EGFR.* (A) Representative end point x-rays for each treatment group (top row), with arrows denoting osteolytic lesion areas.

Corresponding 3D micro-CT images (bottom row). n=10 animals per treatment group. (B) Kaplan-meyer survival curve demonstrating significant increased survival in the shEGFR-MDA-231 injected animals, p<0.001. n=10 animals per group. (C) Osteolytic lesion area was measured using ImageJ software from x-ray images. n=10 mice, p<0.01. PAR34-treated animals required sacrifice at the 3-week time point due to maximum allowable lesion areas and pain scale (per our animal protocol). (D) Micro-CT bone volume analysis of tibiae in all treatment groups. 700 sections were analyzed per tibia. p=not significant.

Figure 4. *Histomorphometric analysis of tumor bearing bones.* (A) Representative images of H&E stained tibiae from each treatment group. Tumor region outlined in white, BM = bone marrow, T=Tumor. (B) Histomorphometric tumor volume analysis on H&E stained tibia sections. Care was taken to measure the same size tissue volume on each section. *p<0.05 and **p<0.01. (C) Osteoclast counts of TRAP stained slides from each treatment group. p=not significant, n=10 mice per group.

Figure 5. *Activated osteoclast measurement by bone marrow and cancer cell co-culture.* (A) Co-cultures of mouse bone marrow with MDA-231, shControl, or shEGFR-MDA-231 cells were TRAP stained to identify active osteoclasts. Four random fields were counted from two separate wells for each co-culture. **p<0.01. (B-D) Co-cultures of mouse bone marrow with MDA-231 cells, (C) bone marrow only, or (D) bone marrow with shEGFR-MDA-231 cells were treated with AREG ligand, PAR34 antibody, Control IgG antibody, or a combination of ligand with antibody as noted. Wells were TRAP stained to identify active osteoclasts, and four random fields were counted form two separate wells for each treatment. **p<0.01, ***p<0.001. (E) bone marrow only or co-cultured with MDA-231 cells were treated with 1 μ M gefitinib or DMSO control for 3 days followed by TRAP staining for active osteoclasts. ***p<0.001

Figure 6. *shEGFR-MDA-231* cells produce smaller tumors in the mammary fat pad. (A) Tumor volume measurement for mammary fat pad tumors grown from injection of shControl, PAR34 treated shControl cells, or shEGFR-MDA-231 cells. PAR34 treated animals were administered 10mg/kg/week of PAR34 by intraperitoneal injection. Tumor measurements were taken three times per week. **p<0.01, n=6 mice per group. (B) Upon sacrifice, tumor masses were assessed. **p<0.01, n=6 mice per group. (C) Paraffin-embedded tumors were stained with anti-CD31 antibody for vessel formation (top row), black arrows denote areas of vessel staining. Ki67 staining (middle row) was examined for cellular proliferation. shEGFR-MDA-231 tumors contained large regions of necrosis, as seen in Necrosis in the bottom row. T=tumor, N=necrotic region. No necrosis was observed in shControl or PAR34 treated tumors. Vessel and proliferation counts, as well as percent changes of necrotic regions are noted in Table 1. n=6 animals per treatment group. Magnification bars, CD31 and Ki67 = 100 μ m. Necrosis = 1mm.

Figure 7. *MCSF-1* and *MMP-9* decrease with EGFR inhibition. (A) anti-VEGF probed western blot for MDA-231, shControl, and shEGFR-MDA-231 extracts, β -tubulin used for loading control. (B) Media was harvested from shControl or shEGFR-MDA-231 cells and analyzed for MCSF-1 by ELISA, *p<0.05. Measurements were obtained from two separate cultures, and performed in triplicate. (C) MDA-231, shControl, SUM149, or NS2TA1 cells were treated with the tyrosine kinase inhibitor PD153035 (10 μ g/mL) compound for 6 hours or PAR34 (10 μ g/mL) for 24 hours before media harvest for MCSF-1 ELISA, *p<0.05 and **p<0.01. Measurements were obtained from two separate cultures, and performed in triplicate. (D) anti-MMP9 antibody probed western blots for shControl, MDA-231, SUM149, or NS2TA1 cell extracts treated with PD153035 (10 μ g/mL) compound for 6 hours or PAR34 (10 μ g/mL) for 24 hours. shEGFR-MDA-231 cells were untreated. anti- β -tubulin used as loading control.

Supporting Information Legends

Figure S1. *Model of inhibition of autocrine and paracrine EGFR signaling within the bone environment.* (A) In an uninhibited situation, cancer cells produce and cleave AREG to act in autocrine or paracrine signaling. Autocrine EGFR signaling can activate the expression of paracrine factors such as PTHrP that can directly stimulate the PTH receptor on osteoblasts and this increases RANKL production and osteoclastogenesis. In addition, stimulation of the PTH receptor induces AREG-EGFR signaling on the osteoblast, leading to increased RANKL accessibility and osteoclastogenesis. Finally cancer cell derived AREG can stimulate the EGFR on the osteoblast in a paracrine manner resulting in increased RANKL accessibility and osteoclastogenesis (B) shEGFR knockdown in cancer cells will decrease autocrine signaling and AREG-EGFR signaling in the endosome, in turn decreasing PTHrP levels. Decreased PTHrP secretion will lead to decreased osteoblast RANKL production, and a decrease in osteolysis. However this should not prevent cancer cell derived AREG from stimulating the EGFR on osteoblasts. (C) PAR34 inhibition of AREG binding the EGFR on cancer cells will decrease PTHrP secretion, and thus decrease RANKL production by the osteoblast. PAR34 may also inhibit cancer cell and autocrine AREG from stimulating the osteoblast EGFR thus reducing RANKL accessibility and osteolysis.

Figure S2. *PAR34 inhibition on bone environment.* (A) Female athymic nude mice aged 3-4 weeks were treated with weekly intraperitoneal injection of PAR34 antibody at 10mg/kg or an equal volume of sterile 0.9% saline as vehicle. Left column, parraffin-embedded tibiae were TRAP stained for active osteoclasts. Active osteoclasts were counted in the primary spongiosum directly under the growth plate. Arrows denote positively stained osteoclasts. Right column, microCT images were reconstructed from the secondary spongiosum, and denote changes in

trabecular bone. For both TRAP staining and microCT analysis, n=10 mice per group. Magnification bar = 170 μ m. (B) MDA-231 or S1 cells were treated with AREG ligand with or without PAR34 antibody, and compared to PAR34 inhibition with EGF ligand treatment. Cell lysates were resolved on 8% SDS-PAGE gels before membrane transfer and probed with the corresponding tyrosine phosphorylated antibodies.

Figure S3. *Gefitinib treatment of bone marrow co-cultures.* Mouse bone marrow (BM) was cultured alone or co-cultured with MDA-231 cells followed by treatment with 0.5 μ M, 1.0 μ M, or 5 μ M of EGFR kinase inhibitor gefitinib for three days. Osteoclasts were counted after TRAP staining from three random fields from two separate wells. *** p<0.001. & denotes all cultured cells in wells were dead after 5 μ M gefitinib treatment.

Table S1. MicroCT and histomorphometry measurements of PAR34 treated, non-tumor bearing tibiae.

Materials and Methods S1. Supporting Materials and Methods.

Tables

Table 1: Histological Tumor Analysis

	Necrosis % Necrotic Tumor Area (mm ²)	Fold Increase	Ki67 Number Cells	Fold Increase	CD31 Mean Vessel Count	% Reduction
MDA-231	16	N/A	31 ± 6	N/A	22 ± 3	N/A
PAR34	19	1.2	34 ± 7	1.09	20 ± 2	9.1
shEGFR-MDA-231	29	1.9*	32 ± 11	1.03	7 ± 2	68.2***

Materials and Methods S1

Pharmacologic reagents

Mouse EGF was purchased from Sigma. Recombinant human ligands for TGF α , HB-EGF, EGF, and AREG, as well as Normal Goat IgG, were purchased from R&D Systems (Minneapolis, MN). PAR34 clone was a gift from PDL BioPharma, and was harvested from mouse hybridomas, purified over a protein A column, and dialyzed into sterile 0.9% saline for injection. PD153035 was purchased from Tocris (United Kingdom). VEGF antibody was purchased from AbCam (Cambridge, MA). MMP-9 antibody was purchased from Santa Cruz Biotechnology. PTHrP (1-34) ligand was purchased from Bachem. EGFR, ErbB2, ErbB3, and Erbb4 antibodies were purchased from Santa Cruz Biotechnology. Gefitinib was purchased from Tocris Bioscience (Ellisville, MO).

Ligand and MCSF-1 ELISA assays

MDA-231 cells were grown to confluence in a 12-well dish and serum starved overnight. Conditioned media was collected and cleared by centrifugation for 10 minutes at 4°C. To measure concentration of ligand attached to cell membrane, cells were harvested in ligand extract solution (1M tris-HCl, 0.5M EDTA, 10% TritonX-100, protease/phosphatase cocktail) and cleared through centrifugation for 10 minutes at 4°C. Ligand concentrations (EGF, AREG, HB-EGF, Betacellulin, TGF α) were measured using the manufacturer's instructions for the respective ligand DuoSet ELISA kit (R&D Systems). For MCSF-1 ELISAs, cells were treated for 6 hours with PD153035 or 24 hours with PAR34. After various time points, media was harvested from the wells and assayed per the manufacturer's instructions. Samples were assayed in triplicate.

Analyses of EGFR phosphorylation and expression

We adapted previously published procedures for assaying EGFR ligand stimulation by assessing tyrosine phosphorylation [1]. Briefly, cells were plated on a 100mm dish and grown to confluence. Cells were incubated on ice for 30 minutes, washed with ice cold PBS and treated with ligand for 7 minutes. Cells were lysed and incubated on ice for 20 minutes. Lysates were cleared by centrifugation at 13k rpm for 10 minutes at 4°C, and transferred to a fresh tube.

Concanavalin A-sepharose beads were used to precipitate glycoproteins (which include ErbB receptors) from cleared cell lysates. Precipitates were resolved by SDS-PAGE on a 7.5 % polyacrylamide gel and electrotransferred to PVDF membrane (BioRad). Blots were probed using an anti-phosphotyrosine mouse monoclonal antibody (Upstate Biotechnology) or an anti-phosphospecific rabbit monoclonal antibodies (Cell Signaling Technology). Primary antibody binding was detected using a goat anti-mouse or goat anti-rabbit antibody conjugated to horseradish peroxidase (Kirkegarrd and Perry Labs Inc) and enhanced chemiluminescence (Santa Cruz). Immunoblots were then stripped and reprobed with an anti-EGFR rabbit polyclonal antibody (Santa Cruz) and detected as described above.

RNA isolation and quantitative real-time reverse transcription PCR (Q-RT-PCR)

Total RNA was prepared using the mini RNA isolation II kit from Zymo Research Corporation according to the manufacturer's instructions. Reverse transcription (RT) and quantitative real-time PCR (QRT-PCR) was performed as previously described [2]. Quantitative real-time PCR (QRT-PCR) was performed using DyNAamo HS SYBR Green qPCR master mix (New England Biolabs) according to the manufacturer's

instruction. PCR reactions were performed in a DNA Engine Opticon System (MJ Research Inc), primers and temperatures are listed in [3].

Immunohistochemistry

Histological tumor sections were rehydrated through alcohol gradation, followed by antigen retrieval for 20 minutes in boiling Tris/EDTA pH 9.0. Sections were blocked in 10% serum for 2 hours, followed by primary antibody for CD31 or Ki67 at 4°C for 24 hours. Sections were then incubated in peroxidase blocking solutions (3% H₂O₂ in TBS) for 15 minutes. Secondary biotinylated-antibody was added for 1 hour at room temperature (Vector Laboratories). Primary antibody was detected using HRP-Streptavidin and DAB peroxidase (both Vector Laboratories). CD31 stained sections were counterstained with hematoxylin, Ki67 slides were not counterstained, and all slides were dehydrated and cleared through xylene before mounting.

Histomorphometry

Bones: Histomorphometry was performed using a Leica DM2500 microscope, fitted with Q-imaging Micropublisher Camera (W. Nuhsbaum Inc., McHenry, IL). Histomorphometric analysis was performed with Bioquant OsteoII 2010 software (Bioquant image analysis corporation, Nashville, TN). Tumor volume was measured on H&E stained bone sections. Osteoclasts that were at the tumor-bone interface were counted at 20X on the tumor-bearing bones, and reported as osteoclasts per tumor bone interface. Osteoclasts in non-injected mice given therapeutics only, were counted in the trabecular region directly underneath the growth plate and reported as osteoclasts per bone surface [4,5]. Care was taken in measuring the same size region for each bone.

Tumors: Ki67 tumor counts: Four 10X images were randomly captured from the cortex of each tumor avoiding the necrotic centers. From each of these images all the labeled cells in 0.1 mm^2 area were counted and a mean number of positive cells for each tumor generated. Counts from each of the shEGFR-MDA-231, shControl, and PAR-34 treated tumors were averaged. Differences in counts among the tumors were not significant.

Necrosis: 10X images were obtained for each H&E stained tumor. Using ImageJ (NIH) the entire tumor area was measured and compared to only the necrotic areas of each tumor. Percent necrosis was calculated.

MTT Assay

500 cells per well were plated in a 96-well dish, in quadruplicate wells per cell line, and allowed to sit overnight in a $37^\circ\text{C}/5\%$ CO₂ incubator. PAR34 or control IgG was plated at 10 $\mu\text{g}/\text{mL}$ when specified. MTT measurements were taken on days 1, 3, 5, and 7 after plating. MTT working solution was used at 1mg/mL MTT in cell culture medium. On days of measurement, 50 μL of MTT working solution was added to each well and incubated for 4 hours at $37^\circ\text{C}/5\%$ CO₂. Medium was removed and 150 μL DMSO added to each well and read on a 96-well plate reader at 600nM.

Migration Assays

BD BioCoat™ Control Inserts were purchased from Becton Dickinson, and used per manufacturers instructions. For each insert, 1×10^5 cells per well were plated and allowed to migrate or invade for 24 hours as in [6]. After 24 hours, each insert was fixed and stained using the Hema3 Stat Pack (Fisher Scientific) per manufacturer's instructions, and allowed to dry overnight. The next day, each insert was carefully cut from the plastic insert using a scalpel blade, and placed on clean microscope slides sealing with

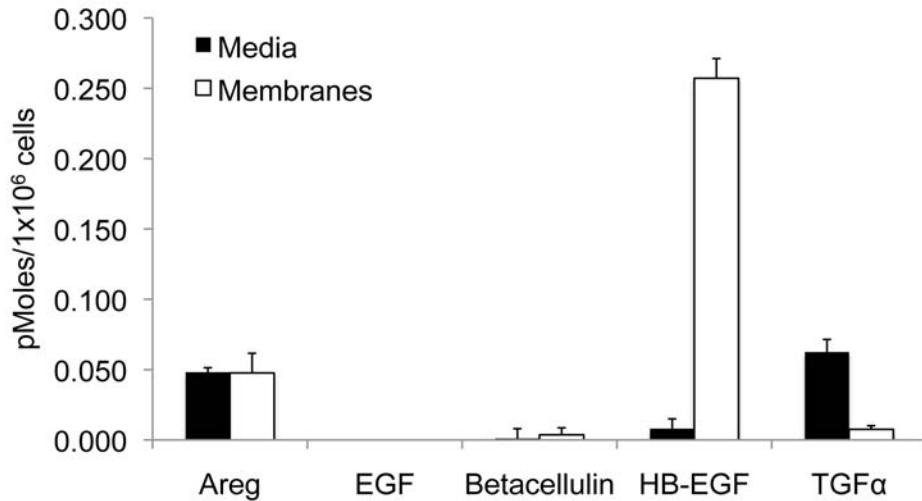
microscope oil. Assays were performed in duplicate migration chambers, with four random images taken per chamber.

References

1. Gilmore JL, Riese DJ, 2nd (2004) secErbB4-26/549 antagonizes ligand-induced ErbB4 tyrosine phosphorylation. *Oncol Res* 14: 589-602.
2. Gilmore JL, Scott JA, Bouizar Z, Robling A, Pitfield SE, et al. (2008) Amphiregulin-EGFR signaling regulates PTHrP gene expression in breast cancer cells. *Breast Cancer Res Treat* 110: 493-505.
3. Cho YM, Lewis DA, Koltz PF, Richard V, Gocken TA, et al. (2004) Regulation of parathyroid hormone-related protein gene expression by epidermal growth factor-family ligands in primary human keratinocytes. *J Endocrinol* 181: 179-190.
4. Dunn LK, Mohammad KS, Fournier PG, McKenna CR, Davis HW, et al. (2009) Hypoxia and TGF-beta drive breast cancer bone metastases through parallel signaling pathways in tumor cells and the bone microenvironment. *PLoS ONE* 4: e6896.
5. Mohammad KS, Javelaud D, Fournier PG, Niewolna M, McKenna CR, et al. TGF-beta-RI kinase inhibitor SD-208 reduces the development and progression of melanoma bone metastases. *Cancer Res* 71: 175-184.
6. Kwon YJ, Hurst DR, Steg AD, Yuan K, Vaidya KS, et al. Gli1 enhances migration and invasion via up-regulation of MMP-11 and promotes metastasis in ERalpha negative breast cancer cell lines. *Clin Exp Metastasis* 28: 437-449.

A

Figure 1



B

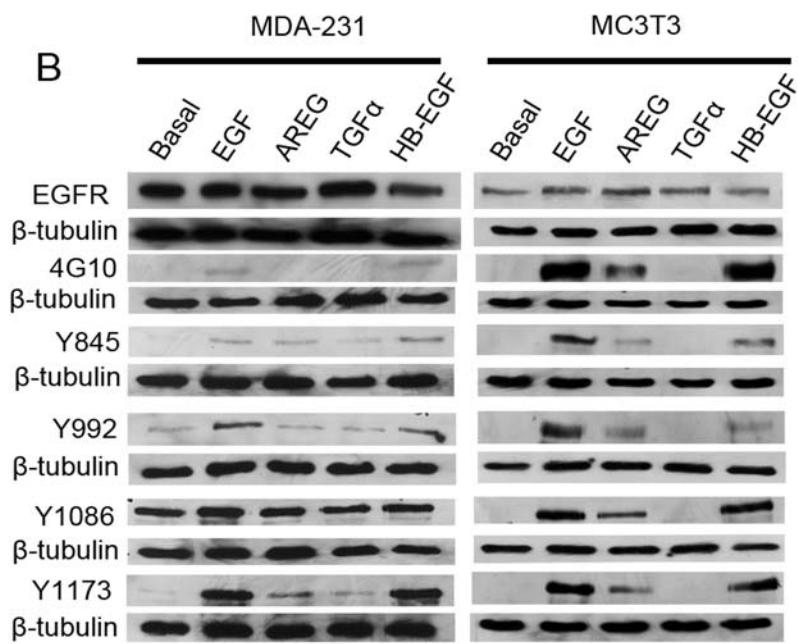


Figure 2

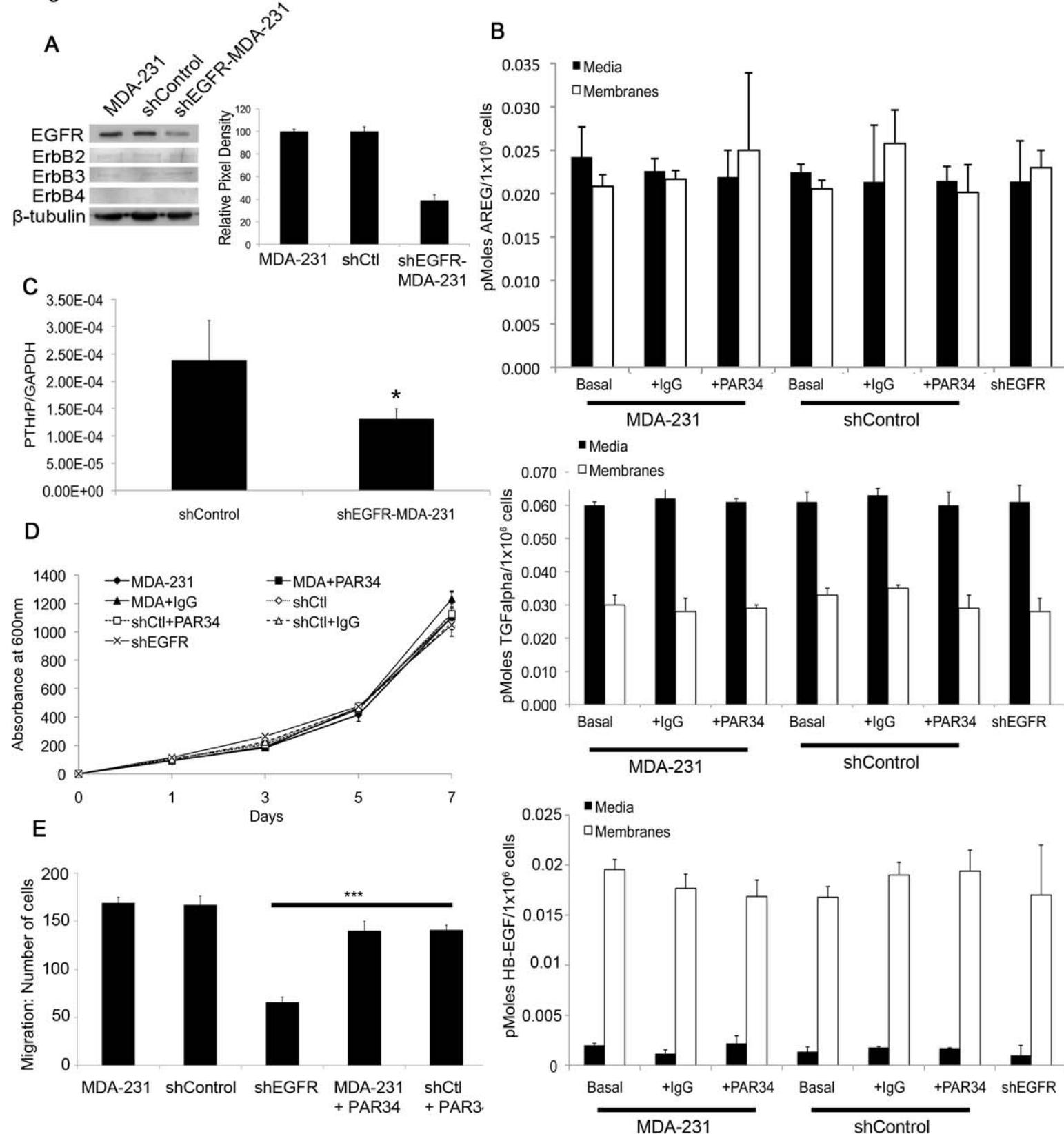
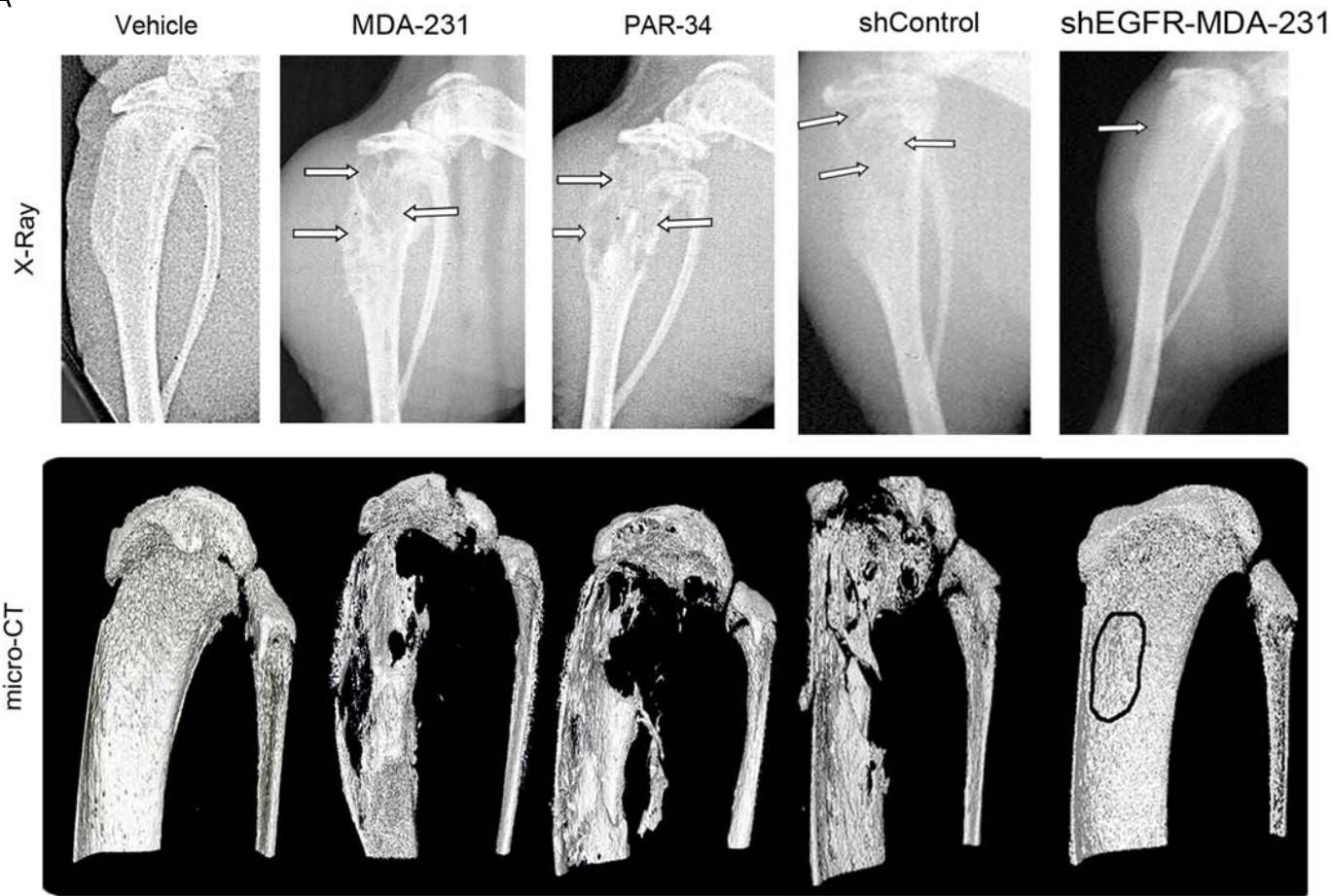
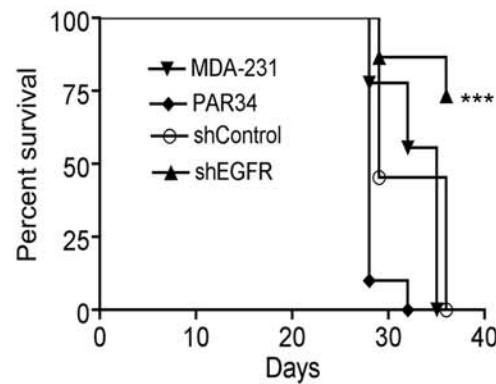


Figure 3

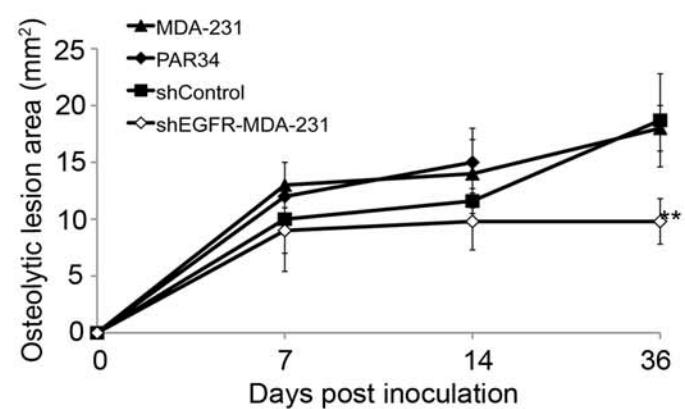
A



B



C



D

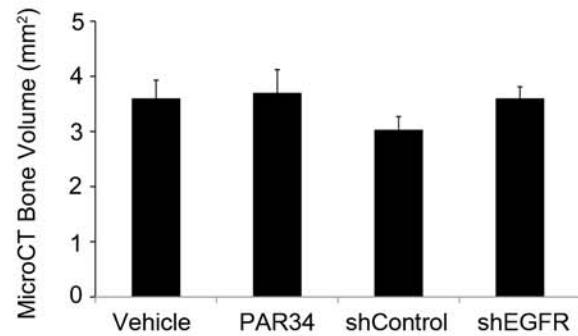
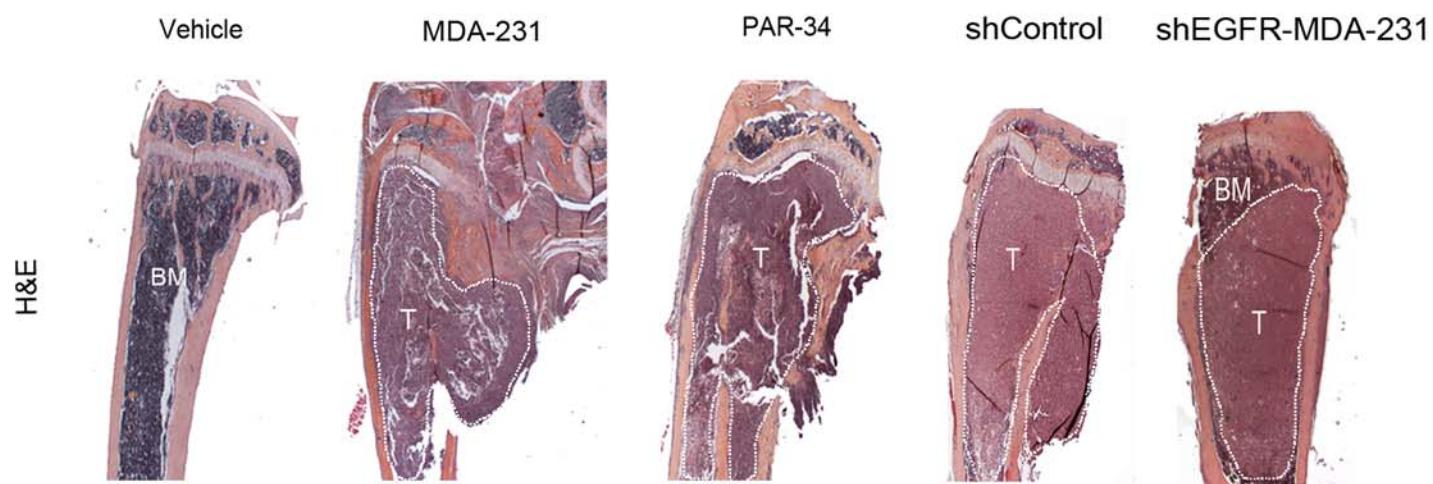
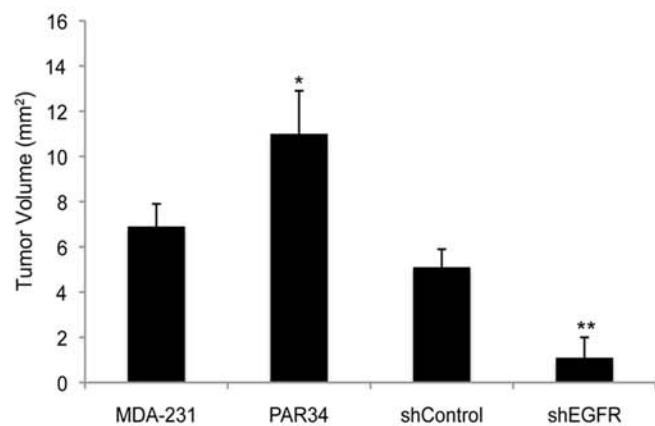


Figure 4

A



B



C

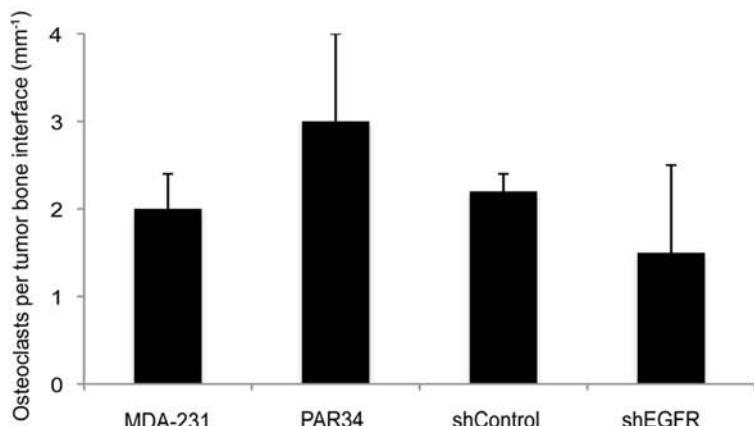
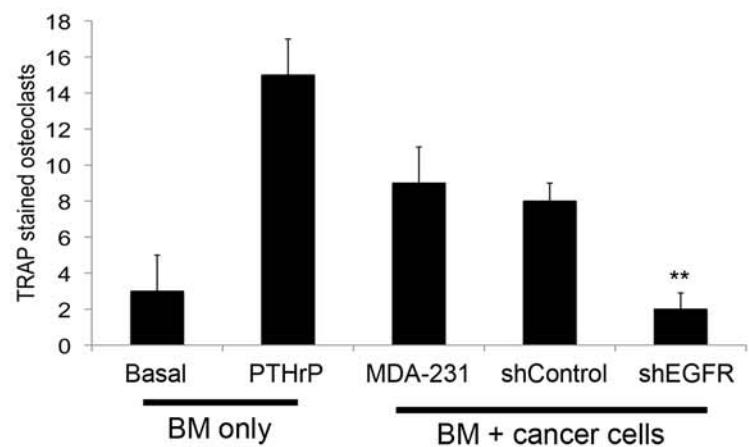
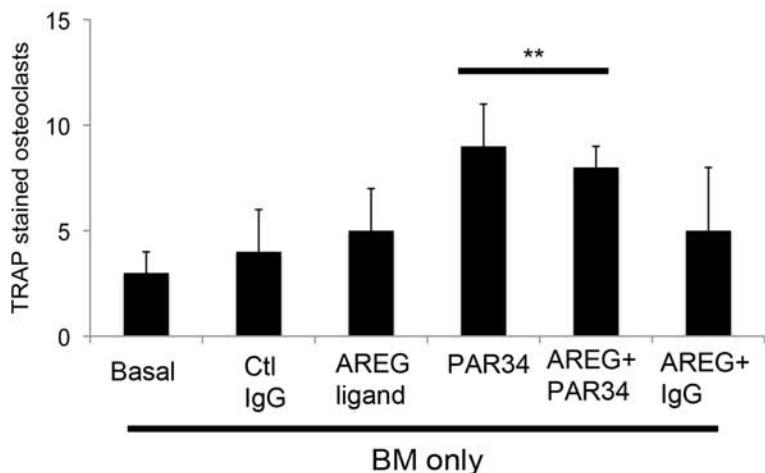


Figure 5

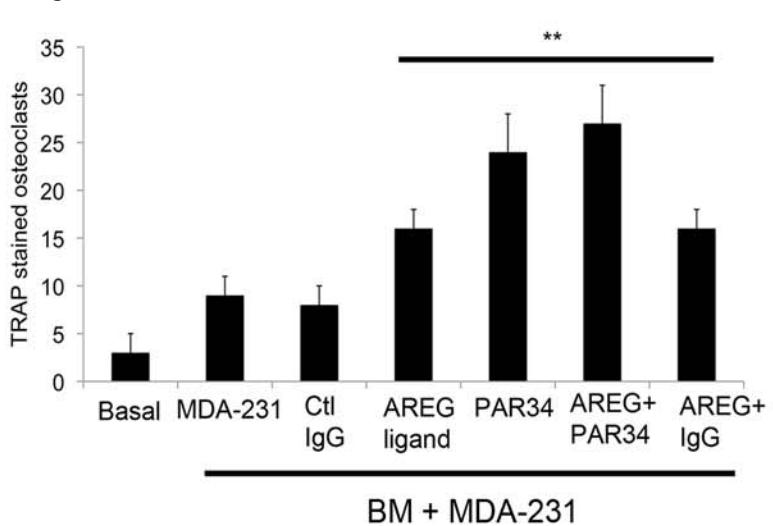
A



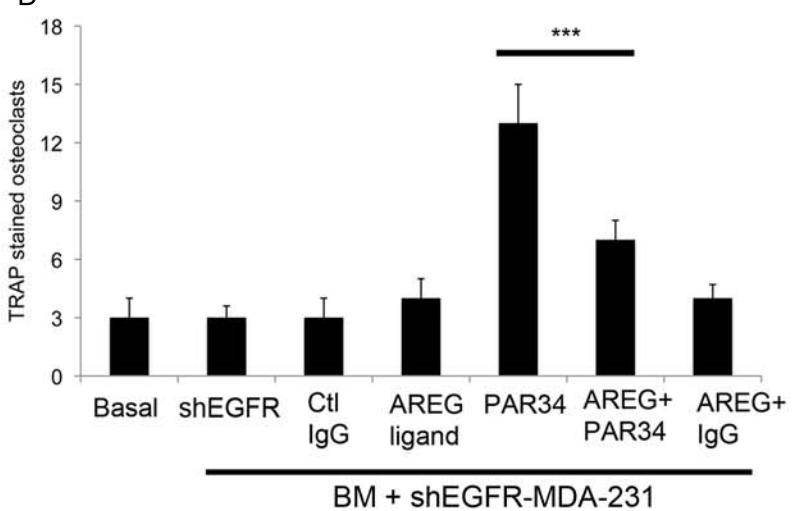
B



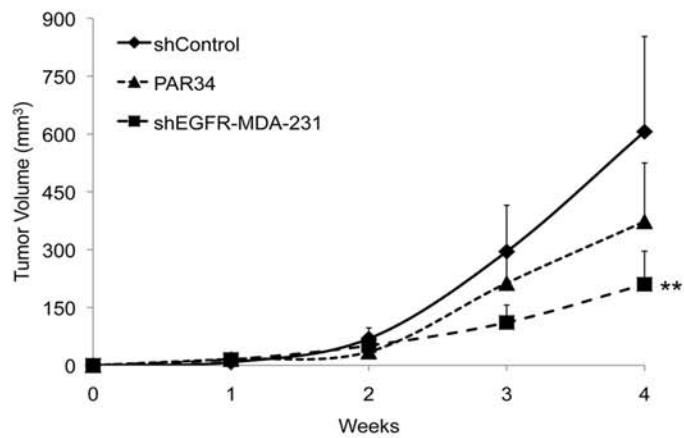
C



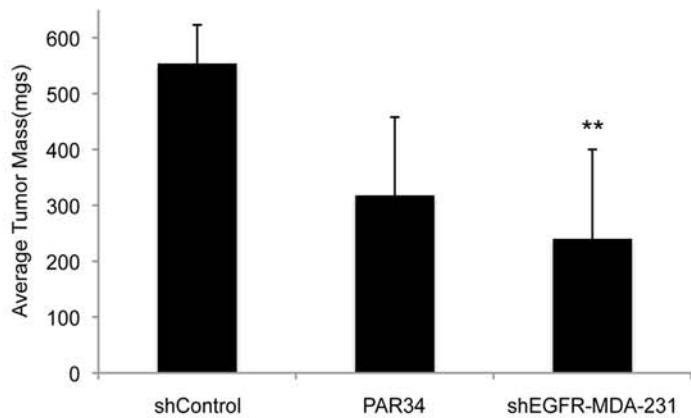
D



A



B



C

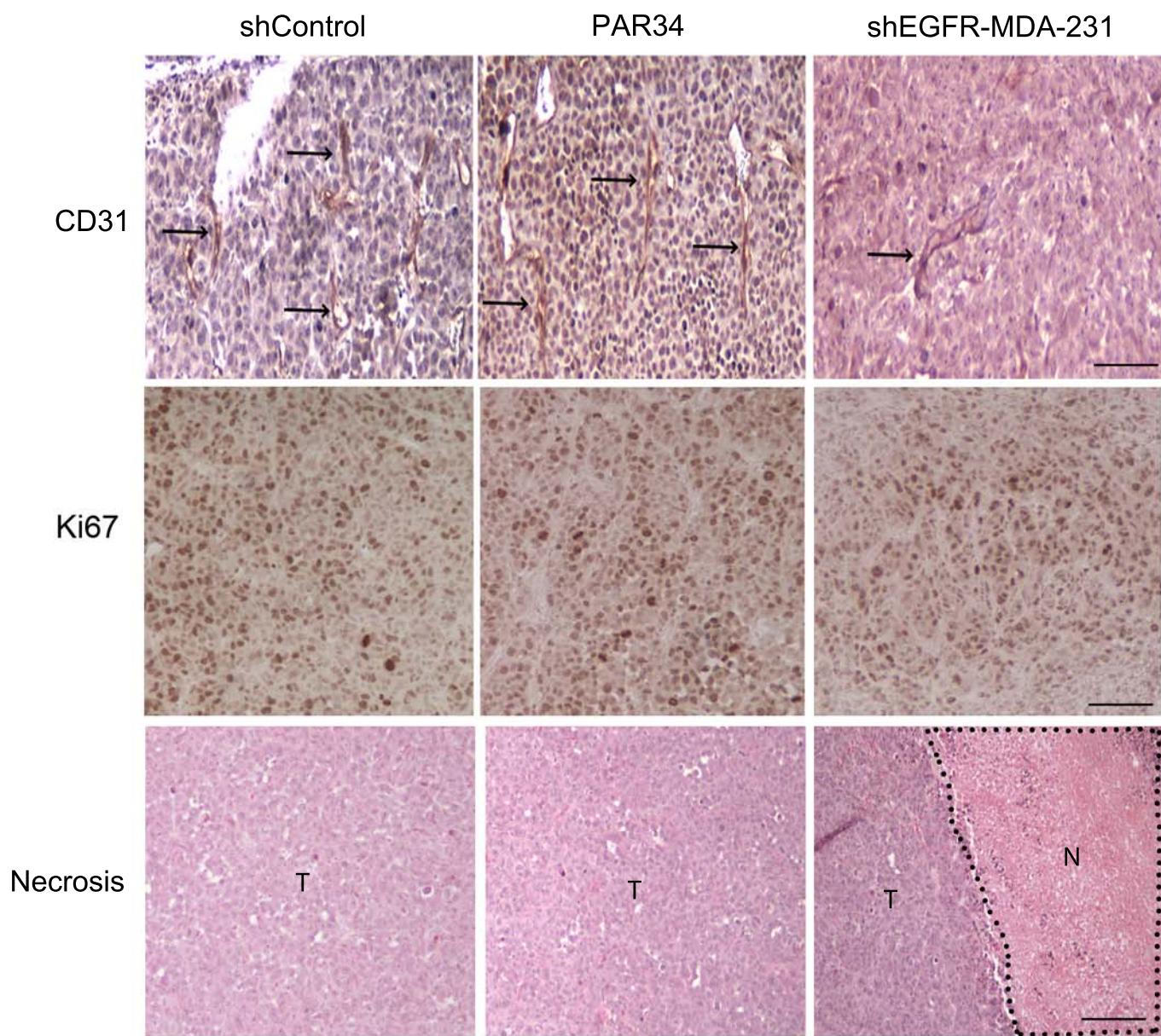
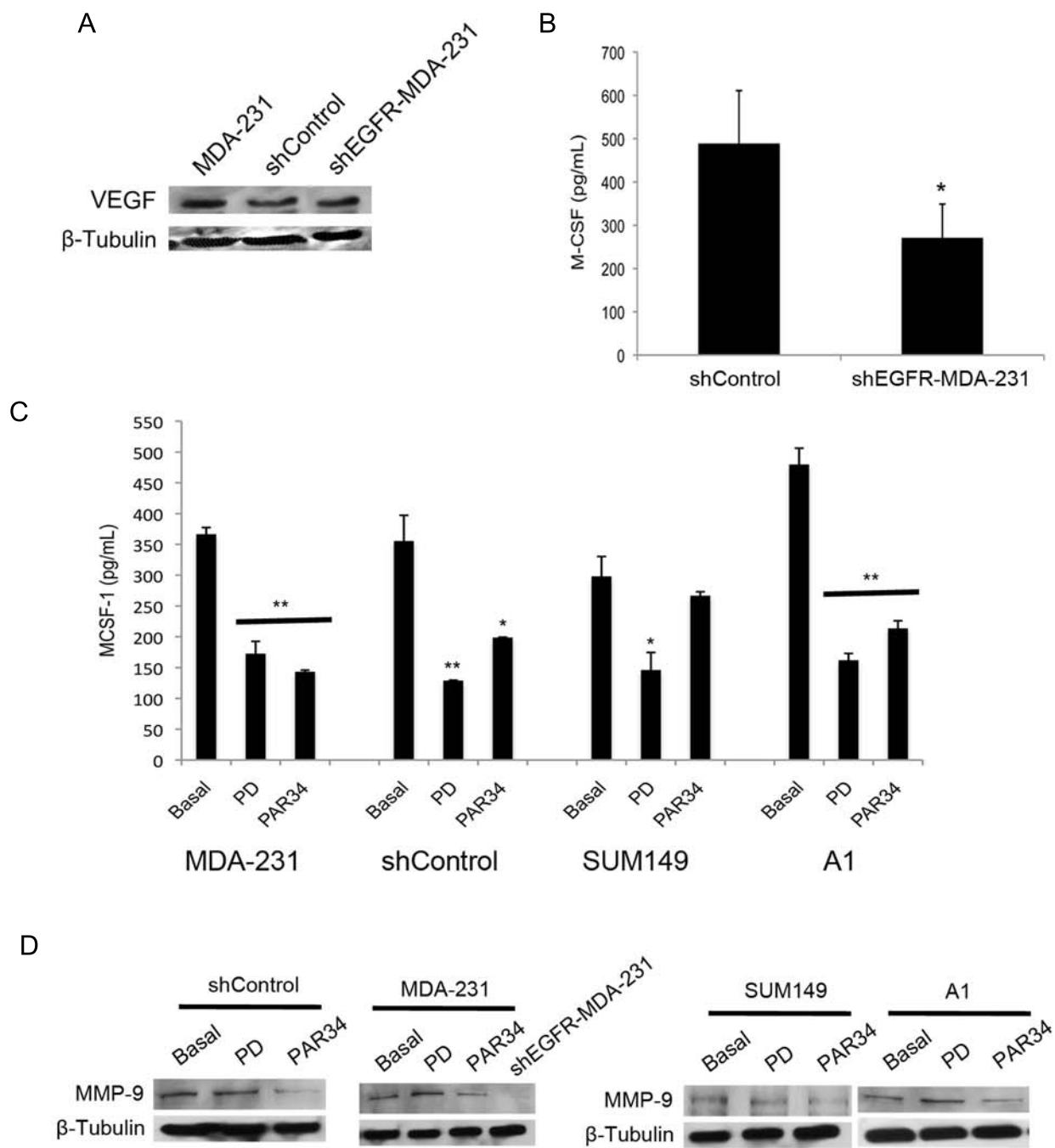


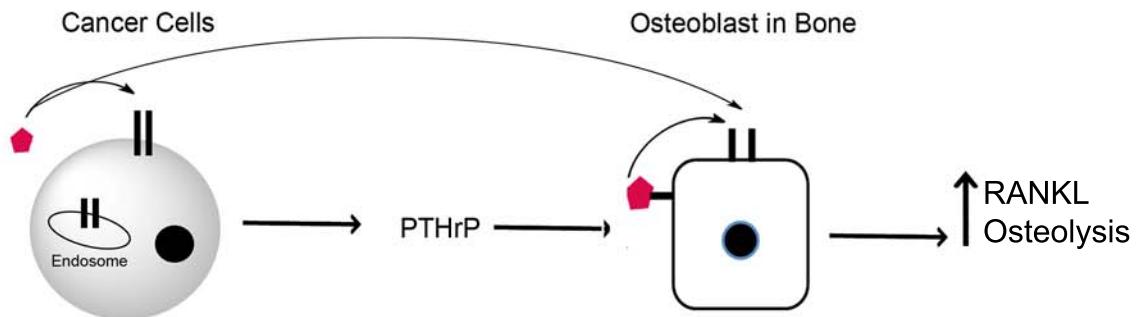
Figure 7



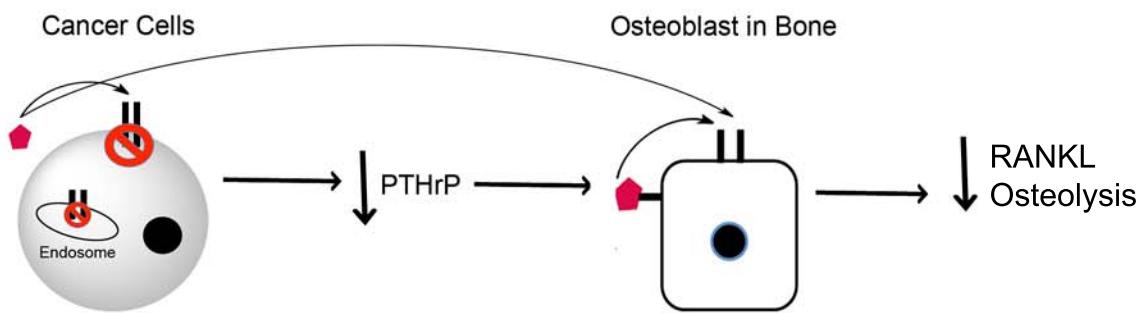
Supplementary Figure 1

II EGFR
♦ AREG ligand

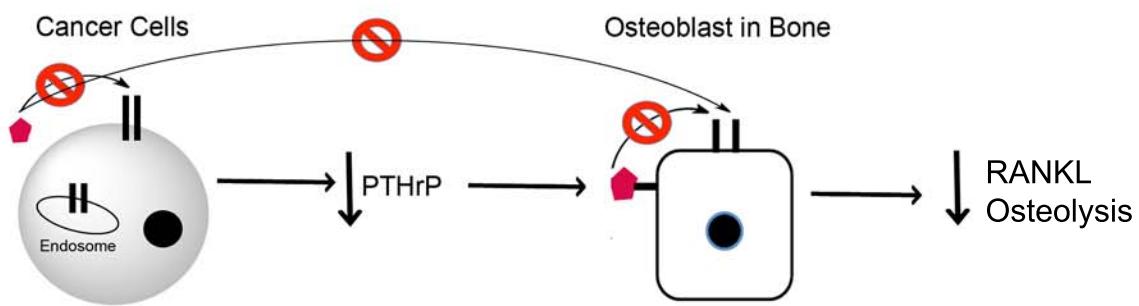
A: Uninhibited



B: shEGFR knockdown



C: PAR34 inhibition

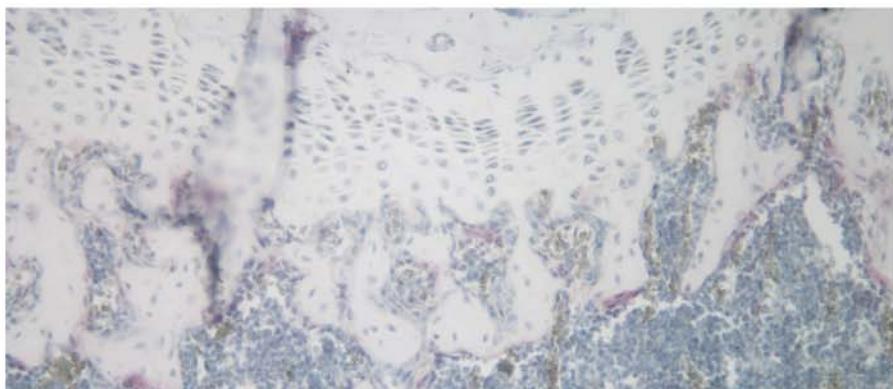


Supplementary Figure 2

A

TRAP stain

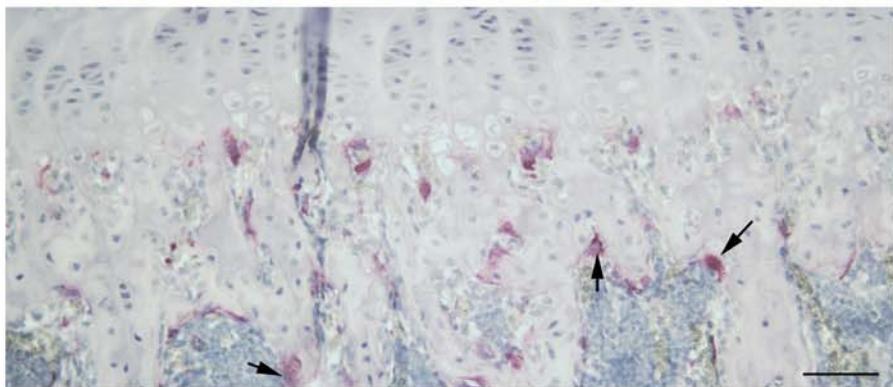
PBS
Control



MicroCT

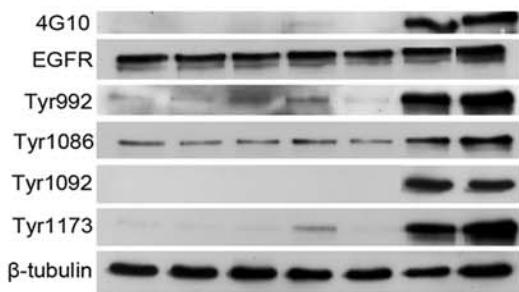


PAR34 Ab

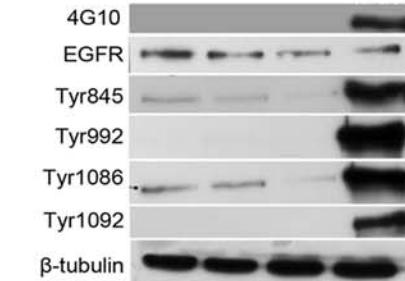


B

MDA-231



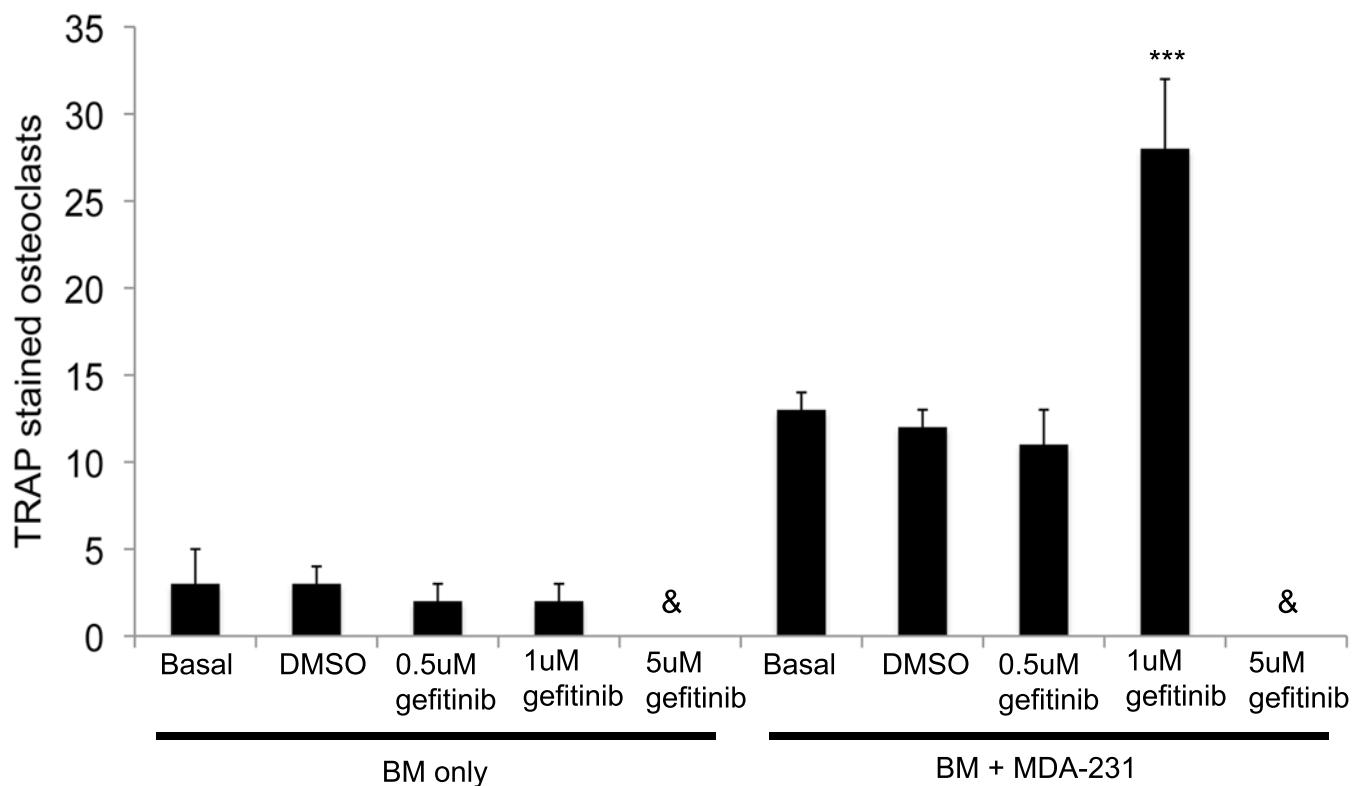
S1



	-	+	-	+	-	+	-
IgG	-	+	-	+	-	+	-
PAR-34	-	-	+	-	+	-	+
Areg ligand	-	-	-	+	+	-	-
EGF ligand	-	-	-	-	-	+	+

	-	+	+	-
Areg ligand	-	+	+	-
PAR-34	-	-	+	+
EGF ligand	-	-	-	+

Supplementary Figure 3



Supplementary Table 1: Impact of PAR34 treatment on bone

	Control	PAR34 (10mg/kg/week)
Bone Volume [^] (mm ³)	3.60 \pm 0.33	3.70 \pm 0.42
Trabecular thickness [^] (mm)	0.06 \pm 0.003	0.06 \pm 0.004
Trabecular number [^] (1/mm)	0.63 \pm 0.12	0.91 \pm 0.14
Trabecular separation [^] (mm)	0.49 \pm 0.04	0.47 \pm 0.03
Trabecular pattern factor [^] (1/mm)	25.77 \pm 3.04	18.72 \pm 2.79 ***
Osteoclasts per bone surface ^{&} (1/mm)	3.14 + 1.23	5.05 \pm 0.93**

** p<0.01

*** p<0.001

[^] obtained by microCT

[&] obtained by TRAP staining

EGFR-Ligand Signaling in Breast Cancer Metastasis: Recurring Developmental Themes

Nicole K. Nickerson¹, Jennifer L. Gilmore¹, Kah Tan Allen¹,
David J. Riese II², Kenneth P. Nephew^{1,3} and John Foley^{1,3,4}

¹*Medical Sciences Program, Indiana University School of Medicine, Bloomington, IN*

²*Harrison School of Pharmacy, Auburn University, Auburn, AL*

³*Indiana University Cancer Center Indiana University School of Medicine, Indianapolis, IN*

⁴*Department of Dermatology, Indiana University School of Medicine, Indianapolis, IN United States of America*

1. Introduction

1.1 ErbB receptors, ligands and signaling

Breast cancer affects nearly 1 out of 9 women worldwide. The quality of treatment for breast cancer has improved to the point that close to 80% of patients in countries with advanced healthcare delivery systems survive the disease (1). Yet over 20% of breast cancer patients succumb to the disease, and the majority of these have metastatic breast cancer cells that occupy and compromise the function of distal organs (1). There has been an intensive effort to improve treatments for metastatic breast cancer. Novel treatment strategies have arisen from the study of the molecular and cellular biology of breast cancer cell lines. These studies have produced a group of agents called targeted therapeutics because they are often directed at a single molecule rather than a general process such as DNA replication or cytoskeletal function. The ErbB family represents a target that is present in breast cancer. Therapeutics to ErbB2 have been used to treat aggressive breast cancer for over a decade with considerable success (2). However, therapeutics that primarily target the EGFR have not been used extensively in breast cancer, and there are some improved agents for the receptor that are just entering the clinic. Recent conclusions from studies of metastatic breast cancer suggest new possibilities for the use of EGFR therapeutics. This review will describe the members of the EGFR signaling family, discuss the cellular context in which they function in development, and correlate this with the biological role of these molecules in breast cancer metastasis.

1.2 ErbB family members

The ErbB family consists of 4 receptors: ErbB1 or more commonly called EGFR, ErbB2/Her2, ErbB3 and ErbB4 (3). Signaling is generated when EGFR and ErbB4 bind to their ligands. In contrast, the ErbB2 extracellular binding domain fails to bind any of the 15 agonists, and in ErbB3 the kinase domain is not functional. Upon ligand stimulation, EGFR and ErbB4 receptors can transduce their signals as homodimers or heterodimers; however,

the signal generation from ErbB2 or ErbB3 require heterodimerization with another ErbB family member (3).

The ErbB receptors are stimulated by 15 ligands but the situation is complicated because several of these agonists can bind more than one receptor. The EGFR exclusive agonists are epidermal growth factor (EGF), transforming growth factor alpha (TGF- α), amphiregulin (AREG) and Epigen (Epi) (3, 4). ErbB4 is specifically bound and activated by Neuregulins (NRG) 3, 4, 5 (3, 4). Heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EREG), and β -cellulin (BTC) bind and activate both the EGFR and ErbB4 (3, 4). NRG 1 and 2 binds both ErbB3 and ErbB4 and NRG 1 can bind the EGFR with low affinity (3, 4).

All of the ErbB agonists are synthesized as plasma membrane bound integral membrane proteins (5). In some cases, the transmembrane ligands stimulate ErbB signaling on adjacent cells through a juxtracrine mechanism which may mediate the stromal-epithelial interactions (6) (7). Most ErbB signaling requires proteolytic cleavage termed ectodomain shedding for the ligand to be released and available to bind receptors that may be on the same cell (autocrine signaling), or on neighboring cells (paracrine signaling) (5). The proteases that mediate the process are from the “a disintegrin and metalloproteinase” or (ADAM) family (8, 9). There are 40 members of the ADAM protein family that function in cell adhesion and ectodomain shedding. These ADAMs are integral membrane proteins in which the extracellular region contains a protease as well as a disintegrin domain that modulates integrin binding (9). ADAMs can be activated by a wide range of stimuli that signal through G-protein-coupled receptors and these signals are often transduced by Src (10). The shedding of AREG, EREG, HB-EGF, Epigen, TGF α and NRG 1&2 is typically catalyzed by the single family member ADAM 17, whereas BTC and EGF are cleaved by ADAM 10 (8). In addition, ADAM 17 cleaves many other cytokines, growth factors, receptors, adhesion molecules and extracellular matrix proteins, suggesting its activity may be a key determinate of cellular behavior (9). Nevertheless, emerging data suggest that EGFR ligands can be shed by other proteases such as the ADAM thrombospondin (ADAMTS) family that is structurally related to the ADAM family, but is secreted and the disintegrin domain is replaced by a thrombospondin domain that binds to matrix (11, 12). Also, it is likely that other metalloproteinases secreted from cells in a paracrine relationship are capable of releasing ligands (13).

Over the past two decades the expression of ErbB receptors, ligands, and their activating proteases in normal breast and breast cancers have been intensively studied. Various mRNA detection methods and immunohistochemistry studies have concluded that the entire ErbB family is expressed in various breast cancers. In fact, it appears the vast majority of the family is expressed in the mammary epithelia (14-16). Since newer therapeutics that target the EGFR are being considered for use in cases of advanced breast cancer, in the rest of this review we will focus on how this receptor is activated and describe its role in development and cancer progression.

1.3 EGFR homodimer signaling

EGF was the first ligand identified and due its abundance in the mouse salivary gland and relative ease of purification from this source (17). EGF has historically been used for receptor binding, signaling, trafficking, and cell fate studies resulting in a model of receptor signaling that is in many ways considered to be the prototype for receptor tyrosine kinases (18-20). The binding of EGF to the EGFR exposes the dimerization arm in the extracellular domain

that permits interaction with another EGFR receptor or heterodimerization with other ErbBs. Ligand binding also induces a conformational change in the receptor that activates the intracellular kinase domain, which in turn can phosphorylate tyrosine residues on the adjacent C-terminal tail of the dimerized ErbB receptor. The 10 phosphorylated tyrosine residues serve as docking sites for adapter proteins or other signal transduction components, resulting in activation of Ras, MAPK, src, STAT 3/5 and PLC γ /PKC and the PI3 kinase-AKT-pro survival pathway. Activation of these signaling pathways by ErbB dimers has profound impact on proliferation, resistance to apoptosis, differentiation, as well as motility/migration associated behaviors. Not all tyrosine phosphorylation of EGFR C-terminal tail results in stimulation of downstream signaling pathways. For example, phosphorylation of the 974 residue triggers endocytosis of the receptor, and phospho 1045 binds to Cbl, mediating ubiquitination of the receptor and subsequent proteosomal degradation (4, 21). Trafficking studies suggest that ~50% of EGF stimulated EGFR is degraded, whereas the remainder is recycled back to the plasma membrane (22). Thus, activation of the EGFR by EGF directly stimulates a broad group of cellular signaling pathways, many of which converge on elements of the ERK/MAPK pathway (3), but this signaling is dampened by receptor turnover. The rapid turnover of the EGF stimulated EGFR is believed to limit stimulation of cellular proliferation, permitting a balance with various differentiation-inducing stimuli present in a normal tissue (3, 22, 23). In cancers, autocrine EGFR homodimer signaling is substantially attenuated, shifting the cell fate balance towards proliferation and survival rather than differentiation, apoptosis and senescence.

1.4 Attenuating EGFR signaling with heterodimerization

Probably the most well understood attenuation of EGFR signaling occurs when the receptor heterodimerizes with the ErbB 2 receptor (24, 25). It is believed that EGFR heterodimerization with ErbB2 frequently occurs in a number of breast cancers (26, 27). Despite being unable to bind ligand, the ErbB2 dimerization arm is constitutively exposed, which allows this receptor to more efficiently dimerize with other liganded ErbB family members (4). The resulting ErbB2 containing heterodimers attenuate EGFR signal transduction in several ways (28-32). First, the affinity of this ErbB2 complex for ligands is enhanced. Second, the ErbB2 phosphotyrosine domains bind most adapter proteins with higher affinity than those of the ErbB homodimers, resulting in more efficient signal transduction. Third, ErbB2/EGFR heterodimers are slowly endocytosed, and are more frequently recycled to the plasma membrane than the EGF stimulated homodimers. By virtue of its strong interactions with adapter proteins and altered trafficking downstream of endocytosis, an EGFR/ErbB2 heterodimer can amplify and extend the duration of EGFR ligand signaling, leading to proliferation and survival at the expense of other cell fates (28-31).

In contrast to the fairly well established understanding of ErbB2 containing heterodimers, there have been few studies on the EGFR heterodimerized with ErbB4 or ErbB3. Co-immuno precipitation experiments have confirmed the presence of the ErbB4/EGFR in a lung epithelial cells and type II pneumocytes; however, the specific function of this complex was not determined (33, 34). Co-expression of ErbB4 and EGFR plasmids in model NIH 3T3 fibroblasts or CHO lines, provided evidence of dimerization of these receptors, and suggested that this complex could induce cellular transformation in the presence of EGF or NRG1. Further analysis of the CHO system found that the ErbB4/EGFR heterodimer

specifically induced B-Raf kinase activity, which was speculated to induce transformation by increasing the activity of the ERK/MAPK pathway (35). Recently, ErbB3/EGFR heterodimers have been identified in pancreatic cancer cell lines (36, 37). It appeared that the ErbB3/EGFR complex may be a more effective stimulus of proliferation in pancreatic cancer cell lines than EGFR homodimers (36). Additionally, these studies suggest the ligand AREG is able to stimulate activity of the ErbB3/EGFR heterodimer (36, 37). Unfortunately, the comprehensive binding, signal transduction and trafficking studies completed for ErbB2 containing receptor complexes have not been completed for EGFR/ErbB4 or ErbB3 heterodimers. This information, coupled with the identification of the specific cell types and tumors that express heterodimers and the function of these complexes will be important considerations for expanded use of ErbB targeted therapeutics.

1.5 Other EGFR ligands

As studies of receptor binding, conformation, phosphorylation, and trafficking are completed for each ligand, it is becoming clear that each agonist induces signaling that can be viewed as a variation of the basic EGF-EGFR homodimer scenario. This attenuated signaling produced by each ligand has the potential to induce subtle differences in downstream signaling, which would be expected to result in altered gene expression and cellular behavior. In the preceding section, the emerging differences in signaling are detailed for each of the ligands that bind the EGFR.

1.5.1 TGF α

Next to EGF, the most intensively studied ligand has been TGF α . Similar to EGF, TGF α exclusively binds to and activates the EGFR. Binding studies suggest that TGF α binds to the receptor with similar affinity as EGF (32). However, conclusions from structural studies involving ligand-receptor complex data indicates there are subtle differences in the conformation of the extracellular ligand-binding domain (sub domain II) induced by TGF α as compared to EGF (4). It is unclear whether this conformational change induced by TGF α could generate alterations in EGFR kinase activity or accessibility of C-terminal tyrosines. Although not comprehensively studied at this point, some of our early studies with breast cancer cell lines suggest that TGF α does not induce the extensive receptor phosphorylation observed with mouse salivary gland derived EGF (Fig 1). Additionally, it has been long recognized that TGF α induces different trafficking of the receptor than EGF (38). Close to 100% of receptors internalized after TGF α treatment are recycled to the plasma membrane (22). At physiological pH of 7.4 in the extracellular environment, TGF α and EGF have similar binding affinities for the EGFR (22, 38). However, at pH close to 5 such as in the endosome, TGF α has decreased affinity for the EGFR (22, 38). It appears that dissociation of the ligand from the EGFR in the endosome permits the receptor to be recycled back to the plasma membrane where it can be reengaged by ligand. It is thought that the three additional histidines found in the receptor binding domain of TGF α provide a greater sensitivity to pH for agonist-receptor interactions (38). In fact, mutations that add histidines to this region of EGF decreased ligand-receptor binding at low pH (39, 40). Together, the altered ligand induced receptor conformation, phosphorylation and trafficking appear to result in TGF α being a more potent stimulator of proliferation of EGFR expressing cell lines than EGF.

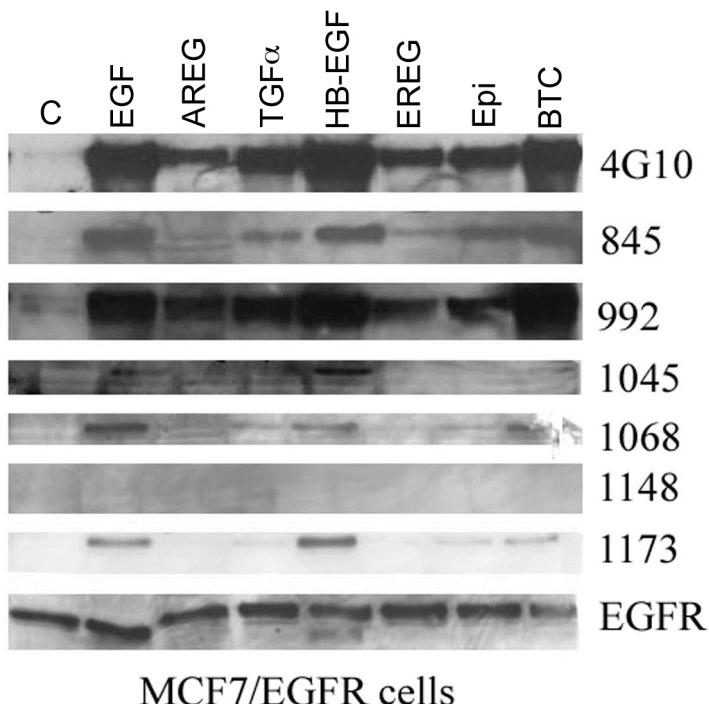


Fig. 1. EGFR phosphorylation after ligand stimulation.

The human breast cancer cell line MCF7 was engineered to overexpress high levels of the EGFR after retroviral transduction. Cells were grown to 80% confluence and placed on ice for 30 minutes. Ligands (R&D, Minneapolis) were applied for 10 minutes to the media and then the media was removed, cells washed and proteins extracted. The EGFR was concentrated with Concanavalin A beads and extracted with Laemmli sample and applied to gels and western blotted with specific antibodies to phosphorylated tyrosines listed on the left. The various ligands used are listed on the top of the figure (C) represents vehicle treated cells.

1.5.2 AREG

The differential impact on breast cancer cell behavior that AREG exhibits compared to EGF has drawn considerable attention to the concept that various EGFR ligands have discrete functions (41-43). Among the ErbB receptors, AREG appears to exclusively bind and activate the EGFR. In addition, the ligand contains a heparin-binding domain N-terminal to the receptor binding region (44, 45). It appears that interaction with heparin-sulfated proteoglycans on the plasma membrane enhances the ability of exogenous AREG to activate the EGFR (46). What has been a matter of controversy has been the relative strength of AREG binding to the EGFR as compared to other ligands. The initial identification of human AREG by Shoyab and colleagues, reported the fully processed ligand isolated from breast cancer cells had reduced affinity for the human EGFR, as compared to salivary gland derived mouse EGF (44). In contrast, subsequent studies with human recombinant ligands

found that AREG has similar affinity for the EGFR as EGF and TGF α (47, 48). Inducing further complexity, additional analyses of ligand receptor interactions have suggested that recombinant AREG does not induce efficient dimerization of the EGFR, as compared to recombinant EGF and TGF α (49). Interestingly, proteolytic processing of AREG in mammalian cells may eliminate the C-terminal portion of the ligand binding domain that is required for high affinity for the receptor (50). In addition, the terminal portion of the receptor binding domain in all other EGFR ligands contains a leucine, whereas a methionine is found in AREG, and this is speculated to reduce affinity for the receptor (50).

More recent studies have focused on the distinct downstream signaling and cellular behavior induced by AREG. Unlike exogenous EGF treatment, AREG stimulation of model cell lines and breast cancer cell lines is unable to induce efficient phosphorylation of many of the tyrosine residues in the C-terminal tail of the EGFR (22, 43, 51, 52) and (Fig. 1). Notably, the Cbl binding 1045 tyrosine residue is not efficiently phosphorylated by AREG and this ligand fails to induce rapid turnover of the EGFR. Trafficking studies indicate that AREG liganded EGFR is rapidly internalized, but then is recycled back to the plasma membrane. In addition, AREG binding to the EGFR is very resistant to acidic pH suggesting that the ligand does not disengage in the endosome as does TGF α (22). It appears that AREG may be unique among the ligands in that it induces EGFR trafficking through Rab 4 and Rab 11 containing endosomes (22, 43). AREG induces prolonged phosphorylation of ERK relative to EGF (41, 52). This altered signaling appears to be the basis of AREG stimulating the loss of cell-cell adhesion and increase motility/migration associated behaviors in breast and other epithelial cells (41, 53). AREG overexpression has also been found to selectively activate interleukin-1 induced NF κ B signaling in breast epithelial cells (41-43).

1.5.3 Epigen

This was the last ErbB family member identified in 2000, and it has not been as intensively studied as other ligands. The ligand activates the EGFR and does not activate ErbB3 or ErbB4 when these receptors are expressed in isolation (54, 55). However, epigen can activate ErbB4 and ErbB3 when these receptors are co-expressed with ErbB2 (54, 55). Epigen appears to have ~100 fold less affinity for the EGFR relative to recombinant human EGF. Not surprisingly, we found that epigen induced modest phosphorylation of breast cancer cells (Fig. 1). The binding of epigen to the EGFR appears to be sensitive to pH similar to TGF α . Modeling suggests that additional histidines in the receptor binding domain are responsible for the dissociation of the ligand from the EGFR at low pH (54, 55). In comparison with EGF, epigen induced significantly weaker ubiquitylation and degradation of EGFR, and once internalized, it appears that the receptor is efficiently recycled to the plasma membrane. As expected for lower affinity ligands, epigen is a more potent mitogen than EGF and displays prolonged MAPK signaling (54, 55).

1.5.4 HB-EGF

Exogenous HB-EGF is a high affinity ligand for the EGFR but it also binds and activates ErbB4 (32). Exogenous HB-EGF does not as robustly stimulate phosphorylation of ErbB4 as it does the EGFR (56). In addition, pro-HB-EGF serves as the diphteria toxin receptor in human cells (6). Similar to EGF, exogenous HB-EGF induces extensive EGFR tyrosine phosphorylation in most cell types studied (22, 56), and we found this to be the case for breast cancer cells (Fig. 1). Upon binding to and activation by HB-EGF, the EGFR is rapidly

endocytosed and the majority of ligand engaged receptors are trafficked to lysosomes and degraded (22). The binding of HB-EGF to the EGFR was resistant to low pH. HB-EGF shows similar effects on cell proliferation and migration to those exhibited by EGF (57).

As its name implies, HB-EGF has a heparin-binding region N-terminal to the EGF domain. This domain has been shown to interact with heparin sulfated plasma membrane proteins such as the tetraspanin, CD9 and the extracellular matrix binding/cell differentiation marker protein CD44 (6, 57). In particular the heparin-mediated interaction between HB-EGF and CD9 appear to be crucial to juxtacrine signaling by the proligand (58). Finally, the associations between the heparin binding domain and cell membrane associated heparin sulfated proteoglycans appear to be crucial to localizing HB-EGF to regions of cell-cell contact. Furthermore, the interaction with these heparin-sulfated proteoglycans prevented proteolytic cleavage of the pro-ligand, whereas exogenous heparin increased shedding of HB-EGF (7, 58). In contrast to the impact of shed ligand, juxtacrine signaling by the pro HB-EGF appears to be antiproliferative (58).

1.5.5 β -cellulin

Exogenous β -cellulin is a high affinity ligand for the EGFR and ErbB4 (32). In general, exogenous β -cellulin phosphorylates the EGFR to a similar extent as EGF in model cell types, and this is what we observed with breast cancer cells (Fig. 1) (22, 59). This exogenous ligand also stimulates total ErbB4 phosphorylation to an extent similar to NRG1, but there may be differences in phosphorylation of specific tyrosine residues (60). Upon binding to and activation by β -cellulin, the EGFR is rapidly endocytosed and trafficked to lysosomes where the majority of it is degraded (22). The binding of β -cellulin to the EGFR was resistant to low pH. Exogenous β -cellulin was slightly less efficient than EGF at inducing proliferation in some specific cell types (60). There have been some reports that β -cellulin binds to heparin and may participate in juxtacrine signaling (61).

1.5.6 EREG

Unlike the other dual receptor ligands HB-EGF and BTC, EREG is a low affinity ligand for the EGFR (32). EREG typically induces much less phosphorylation of the EGFR than EGF in model cell lines (62-65). In breast cancer cells, we found that the epiregulin induced phosphorylation of the various tyrosines on the EGFR to an extent similar to AREG and the low affinity ligand epigen (Fig. 1). EREG is not as effective as NRG or BTC in stimulating ErbB4 phosphorylation (62-65). The ligand appears to preferentially activate heterodimers and efficiently induces EGFR heterodimers with all three other receptors (65). Upon binding to and activation by EREG, the EGFR is rapidly endocytosed, but then is recycled back to the plasma membrane, and its binding to the receptor was resistant to low pH (22). Exogenous EREG was more efficient than EGF at inducing proliferation in some cell types (63, 65). Also EREG does not induce as great of activation of the MAPK pathway as EGF, but the duration of MAPK phosphorylation was increased relative to the prototype ligand (63, 65).

Thus, the expression of specific EGFR ligands could influence the progression of breast cancer in several ways. First, three of the ligands (HB-EGF, BTC and EREG) could induce ErbB4 signaling on breast cancer cells themselves or their microenvironment, whereas the other 4 ligands EGF, TGF α , AREG and epigen would only induce EGFR signaling. Second, if juxtacrine EGFR signaling between breast cancer cells may require interaction with heparin sulfate proteoglycans, only HB-EGF and AREG would be likely mediators of this signaling.

Juxacrine EGFR signaling might be antiproliferative and also only occur in breast cancer that lacked active sheddases that released HB-EGF and AREG. Third, EGFR signaling induced by soluble ligands appears to be dependent on the relative ratio of receptor degradation versus recycling to the plasma membrane. For example, those ligands whose binding leads to rapid receptor degradation (EGF, HB-EGF and BTC) would activate high levels of downstream signal transduction, but this would likely be of short duration. In contrast, the ligands that induce recycling of the internalized receptor to the plasma membrane would produce longer duration EGFR signaling. In the context of autocrine signaling in breast cancer cells, the longer duration EGFR signaling is likely to more efficiently induce mitogenesis. What remains to be determined is whether the duration of EGFR signaling differentially impacts other cellular behaviors relevant to breast cancer progression such as resistance to apoptosis, and the stimulation of invasive/motile behaviors. Finally, in the context of paracrine signaling it is not clear how EGFR turnover influences the supportive functions of stromal and immune cells of the tumor microenvironment.

2. EGFR/ErbB signaling in development

2.1 ErbB signaling and mammary gland development

The majority of research on mammary gland development is performed in the mouse model due to its biologically and histologically similarity with humans, and the power of transgenic knockout murine models (66). Embryonic mammary gland development in the mouse begins around embryonic day 10.5 (E 10.5), where bilateral milk lines are formed from front to hind paws. Between E11.5 and E12.5, five placodes on each milk line develop with eventual epithelial bud formation at each placode. These epithelial buds remain quiescent until E15.5, where minor branching permits the migration of mammary epithelia into the fat pad, and mesenchymal differentiation forms the overlying nipple epidermis. This rudimentary mammary gland will remain quiescent until after birth (67). The majority of mammary gland growth and development occurs in postnatal life during puberty and pregnancy, and ErbB signaling impacts this phase.

The primary hormone that drives post-natal mammary gland development is the nuclear steroid hormone, estrogen. Estrogen stimulates proliferation of luminal cells within the mammary ducts, causing ductal elongation and branching (68). Progesterone also increases cellular proliferation of the mammary ducts, and acts synergistically with estrogen during periods of high hormone levels such as pregnancy (68). Prolactin, a non-steroid hormone released from the pituitary gland, is active in mammary gland development, late in pregnancy, stimulating alveolar development and triggering milk production during lactation (69).

The mouse mammary gland begins pubertal outgrowth between 3-4 weeks of age, and is complete at 8-12 weeks of age. The gland requires both longitudinal ductal growth, as well as ductal branching to fully infiltrate the mammary fat pad. Pubertal growth is directed by the cells within structures called terminal end buds (TEBs), which are a bulbous expansion of the epithelia. The TEBS are found at the distal end of each growing duct, and consist of 3 to 4 cell layers, including cap cells that make up the 'basal' layer and multiple layers of interior luminal-like body cells that line the duct. Signaling within the cells of the TEBS and the surrounding stroma will determine the extent of continued ductal branching (70) (71). From this point, the mammary gland will show minimal growth with each estrous cycle. Upon pregnancy, estrogen and progesterone drive another large spurt of growth resulting

in extensive ductal branching. Progesterone also works with prolactin to signal differentiation of the secretory or alveolar cells throughout the duct system, which produces the large volumes of milk post parturition (72, 73). Termination of lactation will eventually lead to involution, where large-scale apoptosis will eliminate the secretory alveoli and remodel the remaining ducts of the mammary gland, returning the structure to a state similar to that of the virgin gland.

2.1.1 Role of EGFR in mammary gland growth

In virgin mice, EGFR, ErbB2, and ErbB3 are present in the developing ductal structure while there is minimal ErbB4. During pregnancy, this pattern changes to greatly increase expression of ErbB4 in the mammary epithelium, while ErbB4 levels will again regress during lactation and involution (74). Even though three of the receptors are present during growth, there is minimal ErbB phosphorylation observed until ductal morphogenesis begins. During pubertal growth, phosphorylated EGFR and ErbB2 are detected, which suggested that these receptors may mediate the impact of estradiol (E2) on the gland (75).

The EGFR^{-/-} mice die within 8-days after birth and show a wide range of dysfunctional epithelia, but their mammary glands were similar to their wild-type littermates. Transplantation of pre-pubertal glands from EGFR^{-/-} mice into cleared fat pads of wild-type littermates failed to infiltrate the structure, but glands from wild-type mice produced normal ductal systems (76). In contrast, when a purified mammary epithelium from the EGFR^{-/-} mouse was implanted into cleared fat pads with mammary stroma from wild-type mice it produced a normal ductal tree, whereas the opposite combination of wild-type epithelium and EGFR^{-/-} stroma failed to penetrate the fat pad. Thus, postnatal mammary ductal growth is dependent on the presence of the EGFR in mammary stromal fibroblasts. It appears that signaling by the receptor triggers the production of stromal growth factors important to TEBs in ductal elongation (76).

The EGFR ligands EGF, TGF α , and AREG, are found in different locations within the TEB during ductal growth. Using immunohistochemical techniques, TGF α is found exclusively in the basal cap cell layer while the luminal cells express only EGF (70). AREG has been found in both the basal cap cells and the luminal cell layers of TEBs (77). Luekette and colleagues produced knockout mice for each of these ligands separately or as double and triple knockouts. While all three mice null for the individual ligands and their various crosses were fertile, not all had distinct mammary phenotypes. Double-knockout mice for EGF and TGF α , but which contained AREG, displayed normal ductal growth and TEB formation. Mice that were single-knockout for AREG or a triple-knockout for all ligands displayed almost a complete lack of ductal growth into the fat pad at 8-12 weeks (78). In the AREG^{-/-} mice, mammary epithelial failed to fill the fat pad even after multiple pregnancies, strongly suggesting that AREG-EGFR signaling mediated the impact of estrogen on mammary ductal growth. This suggests that EGF and TGF α are dispensable for mammary gland growth, while AREG plays a vital role in glandular development. Recombination grafts indicated that estrogen stimulated pubertal mammary gland growth will not occur without AREG signaling to the stroma (79). The AREG gene is regulated by estrogen receptor alpha (ER α), which apparently accounts for its requirement in postnatal mammary gland development (80, 81). Whether there are any ligand specific effects of the AREG ligand in mammary development have not been explored.

Studies performed with mice deficient for ADAM-17 have shown cardiac insufficiencies, a constellation of epithelial defects and die soon after birth similar to the phenotype of EGFR -/- mice. The ADAM17-/- mice have small, immature mammary glands with minimal branching or ductal growth (82). To verify the requirement of ADAM-17, the defective growth of ADAM-17-null mammary epithelia can be rescued in the presence of exogenous AREG, EGF, or TGF α (82). In conclusion, estrogen induced growth of the mammary pad requires mammary epithelial cell ADAM-17 to shed AREG which then, is necessary to stimulate EGFR signaling in the stromal fibroblasts.

2.1.2 Breast epithelial stem cell and ErbB signaling

Potential mammary gland stem and progenitor cells have been identified using a series of methodologies used to identify the hierarchy of cells that produces that mature hematopoietic system. A single human mammary gland stem cell has been shown to regenerate all the cellular components of the human mammary gland, as well as produce milk proteins in immunocompromised mice (83-86). This work, coupled with mouse work has given rise to an epithelial hierarchy illustrated in figure 2. In this hierarchy, the mammary stem cells give rise to “the common or bipotent” progenitor; the bipotent progenitor gives rise to a luminal progenitor, as well as a cell type that gives rise to mature myoepithelial cells; and the luminal progenitor produces derivatives that ultimately differentiate into mature duct and alveolar cells (83-86).

The reproducible isolation of stem and progenitor cells from mammary epithelia has permitted profiling the various cell types for the expression of the receptors involved in post-natal mammary gland growth and breast cancer progression (85). The subpopulation containing putative mammary multipotent stem cells appear to lack expression of estrogen and progesterone receptors, whereas the EGFR is expressed in ~12% of this fraction. Nearly 50% of the luminal progenitor inclusive population expressed high levels of EGFR. The relatively small subset of the differentiated luminal cell segment (ductal and alveolar cells) express the EGFR (83-86). Of possible significance is that both the mammary stem cell and luminal progenitor population are routinely propagated in a media supplement containing EGF. Whether this implies that propagation of these stem and progenitor cells are dependent on EGFR signaling or simply that there is a requirement for generalized receptor tyrosine kinase activity remains to be determined.

Taken together, the post-natal development of the mammary gland is regulated in large part by the EGFR. EGFR signaling in stromal fibroblasts is required for the estrogen-stimulated invasion mammary epithelium into the stromal fat pad that establishes the adult virgin mammary gland. It appears that the stromal EGFR signaling is mediated primarily by the estrogen-controlled ligand, AREG. Interestingly, the EGFR and its ligands are expressed in the mammary epithelia, but the recombination experiments suggest that autocrine receptor activity in this compartment is dispensable for the establishment of the adult mammary gland. At this time, it is not clear if autocrine EGFR signaling in a mammary epithelial stem or progenitor cells might be required for maintenance of the organ throughout adult life.

2.2 EGFR signaling and cardiac development

Careful reexamination of the EGFR-/- mice along with observations from the Waved-2 mice (these express a mutant form of the receptor with only 10% kinase activity) found defective

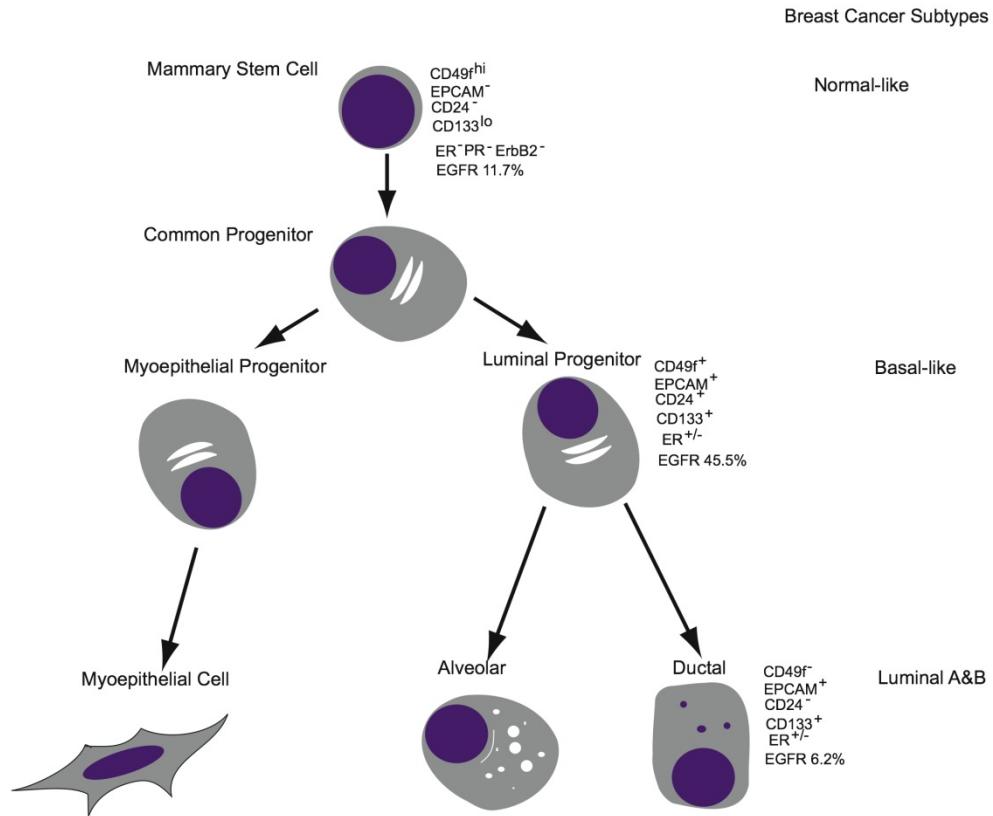


Fig. 2. Hierarchy of mammary epithelial cells.

The various cells of the mammary epithelium and their relationships are represented. To the right, is the expression pattern of the various cell surface markers. Below this is the ErbB and ER α expression along with breast cancer cell types the various cells are related to.

cardiac valve morphogenesis and maturation (94, 96). HB-EGF KO mice also exhibit defective maturation of cardiac valves, suggesting that this may be the relevant ligand that induces EGFR signaling in this process. Also, the HB-EGF-/− cardiac defect was phenocopied in the ADAM-17, suggesting that this protease released the ligand during cardiac development (97).

In the context of cellular behaviors, it appears that the EGFR signaling system plays a role in differentiation. In the EGFR, HB-EGF and ADAM-17 KO mice which have hyperplastic valves it appears that the impact of this signaling is distinct from a proliferation and migration defect observed with the other ErbB knockouts. It is thought that HB-EGF-EGFR signaling decreases BMP expression, the factor which drives cardiac valve maturation, and hence is a differentiation factor (97).

2.3 EGFR signaling and nervous system

In the mouse, EGFR is highly expressed during brain development (E-7 to E-17) and is present on multipotent precursors of both neurons and glia, as well as developing astrocytes and some neurons (98, 99). The initial reports of the EGFR knockout did not identify a nervous system defect. However, reexamination of the KOs with extended post natal survival due to breeding of the knockout allele onto other mouse strains was able to identify brain defects (98). Among the defects identified were smaller or thinner forebrain regions, including the cerebral cortex, olfactory bulb and neocortex (98). Both HB-EGF and TGF α are expressed in portion of the fore brain during late embryonic and early postnatal life (98, 100). Modest histological defects were observed in the prefrontal cortex of mice with a conditional KO of HB-EGF in the forebrain, and these mice displayed behavior and defects in dopamine metabolism that have been observed in schizophrenia (98).

The defects observed in the forebrain of the EGFR-KO mice appear to result from disruption of the cellular interactions required to support neurons. There was substantial neuronal apoptosis in the early postnatal forebrain regions affected (98). However, this occurred in EGFR-expressing and non-receptor bearing neurons. In addition, there was a delay in the appearance of glial fibrillary acidic protein (GFAP) positive astrocytes in the glial limitans and white matter tracks of the fore brain. Although EGFR ligands can stimulate both the proliferation of astrocytes and recruitment of these cells from multipotent precursor cells, the major defect of the knockout mice appears to have a defect in the migration of these glial cells from germinal centers. It is speculated that the delay in formation of contacts between neurons and astrocytes results in a deficiency of trophic support, resulting in neuronal cell death in the forebrain (98, 99).

EGFR expression is high in developing astrocytes, but the receptor is not present in mature astrocytes of the healthy adult brain. Upon injury or disease, EGFR expression is up regulated in reactive astrocytes (101). Reactive astrocytes lengthen processes produce plasma membrane pseudopodia and increase expression GFAP in response to all forms of CNS injury or disease (102). Stimulation of the EGFR on reactive astrocytes results in the upregulation of motility chemokines and extracellular matrix remodeling genes that are likely to contribute to glial scarring (101). Intriguingly, the use of EGFR tyrosine kinase inhibitors reduced nerve loss and lead to greater nerve fiber regeneration in optic nerve crush a model of a glial scarring (101). Thus, EGFR signaling in astrocytes facilitates neuronal survival during development, but receptor activity in reactive astrocytes actually contributes to neuron loss in pathologies.

2.4 EGFR signaling and bone

Bone phenotypes had not been reported in the original characterization ErbB receptor KO mice. However, work on the problem of malignancy-associated hypercalcemia had long established that TGF α increased the formation of bone resorbing osteoclasts in bone marrow

cultures and whole animals (103, 104). The EGFR is expressed on both chondrocytes and cells of the osteoblast lineage in animals and humans (105). However, the function of the receptor was not established until a human EGFR gene-knockin mouse was created (106). This human EGFR transgene had a limited expression in mouse tissues that normally express the receptor, probably due to the presence of the Neo gene in the first intron of the construct. The human EGFR was expressed in the heart and nervous system and provided a rescue of the murine EGFR KO, but the receptor was not expressed in epithelia and bone. The human EGFR knockin mice were growth retarded and the skeletal phenotype appeared to be largely due to premature hypertrophy of the growth plate cartilages. Although routine histology did not reveal defects in the bones, growth of the knockin osteoblasts *in vitro* resulted in the increased formation of calcified nodules, which represent the end point of differentiation for these cells. Thus, in both cartilage and bone, EGFR signaling inhibits differentiation and helps maintain chondrocytes and osteoblasts in a proliferative state. At this point, it is unknown if any other ErbB receptors play a functional role in bone development or physiology.

Further insight into the role of EGFR in bone resulted from a study of global changes in osteoblast gene expression induced by the main serum calcium regulator, PTH. Activation of the PTH receptor on osteoblasts rapidly upregulates AREG mRNA expression 10 to 20-fold, as well as increasing the TGF α and HB-EGF ligands (107, 108). In addition, PTH signaling induces shedding of ADAM-17 controlled ligands in the kidney (109). Further experiments indicated that addition of exogenous AREG to osteoblasts stimulated their proliferation. However ligand-EGFR signaling also inhibited osteoblast differentiation and dramatically decreased mineralization of osteoblast cell lines. Consistent with the role for AREG in stimulating the proliferation of osteoblasts, 4-week-old AREG-knockout mice exhibited less trabecular bone in the tibia than wild type littermates (107). These experiments suggested that EGFR signaling may mediate the impact of PTH on the recruitment and expansion of cells committed to the osteoblast lineage, but excessive signaling by this system could prevent these cells from undergoing terminal differentiation and forming mineralized bone. The inhibition of osteoblast differentiation and subsequent mineralized bone matrix deposition by-EGFR signaling may contribute to the uncoupling of bone formation from the accelerated bone resorption

3. EGFR and breast cancer

3.1 ErbB and EGFR expression in primary tumors

The development of platforms capable of simultaneously evaluating gene expression from a large portion of the genome have lead to identification of gene expression profiles that correlate with various established and some novel classes of breast cancer. These profiles have produced further insights into the impact of ErbB family members in breast cancer progression. Based on these studies, breast cancers are now divided into the following subclasses: ErbB2 amplified, luminal A, luminal B, normal breast-like, and basal (110-112). The ErbB2 amplified, basal and luminal B subtypes had substantially worse prognosis than the normal breast-like and luminal A.

3.1.1 ErbB2 amplified tumors

Among the molecular subclasses of breast cancer, the ErbB2 amplified, has the most well established functional role for an ErbB member in disease development and progression. The ErbB2 amplified tumors typically express ErbB3 and cell line experiments suggest the

ErbB2/ErbB3 heterodimers stimulate proliferation of these cells through the PI3 kinase-AKT pathway (113). Despite the identification of the ErbB2 co-receptor, the precise ligand activating the ErbB3 has not been established. In addition, the correlation between high ErbB2 expression and poor prognosis suggests that ErbB2 contributes to metastasis and how the receptor contributes to these processes, is still under investigation (110-112).

3.1.2 ER+ tumors: luminal A&B

Luminal A tumors express ER α along with GATA binding protein 3, X-box binding protein 1, trefoil factor 3, and other estrogen-regulated genes and high levels of the luminal keratins K8 and 18 (110-112). Luminal B tumors tend to express the above markers at slightly reduced levels, but have an upregulated cassette of genes, including proliferation related genes such as Myb and components involved in DNA replication. There is no specific ErbB family member included in luminal A or B signature. Further evaluations of ER α + tumors have indicated that the majority of these tumors lack ErbB2 and EGFR expression, but close examination of data from microarray and PCR studies suggest there are occasional luminal type tumors that express these receptors (16, 110-112). A large fraction of ER α + tumors also contain ErbB4 (110-112), and there is some indication this receptor may be involved in a reciprocal regulatory loop with ER α signaling (114). Surprisingly, AREG was not in the original gene set that defined ER α + luminal tumors. A follow up interrogation of data that was used to relate disease outcome to cancer subclasses identified a correlation between higher levels of AREG expression, ER α , and the luminal A subclass (115). Also, this analysis indicated that ADAM-17 levels were low in the luminal A class relative to other tumor subtypes. These observations suggest that although most ER α + luminal A breast cancers express AREG, they lack the EGFR; therefore, autocrine signaling by this ligand receptor system should not be present in most of these tumors. Because the tumor cells express low levels of ADAM-17 it is unclear whether AREG could even participate in paracrine signaling between luminal A breast cancer cells and the tumor stroma. There is a possibility that other proteases produced by the tumor cell or microenvironment lead to shedding of AREG by luminal A breast cancers but whether this signaling impacts progression is unclear.

3.1.3 Normal-like breast cancers

The gene expression signature of these tumors clustered with the normal breast samples. These tumors had a signature that was not associated with epithelial cell types. They instead express high levels of collagen receptors, lipoprotein lipase and glycerol-3-phosphate dehydrogenase normally found in stromal cells or adipocytes. High levels of AREG and moderate levels of ADAM-17 were observed in occasional samples from this group of tumors, but the EGFR was absent. At this time there is no evidence that ErbB signaling plays any role in the biology of these tumors (110-112).

3.1.4 Basal tumors

These tumors lack the expression of the estrogen, progesterone and ErbB2 receptor-for this reason they are often called triple receptor negative tumors-and these cancers express some markers consistent with the myoepithelial cells that are in contact with the basement membrane. These tumors express high levels of the epithelial markers kertatin 5 and 14 (basal keratins), P cadherin as well as troponin (110-112). Basal breast cancers are correlated

with poor survival, high rates of distant metastasis and are generally high grade, large tumors. Once the category became established, antibody labeling studies indicated that 50 to 70% of the basal cancers expressed high levels of EGFR immunoreactivity (116). Low levels of EGFR expression is correlated with reduced numbers of distant metastasis (117). These tumors also frequently express elevated levels of TGF α and ADAM-17 (115).

Within basal breast cancers there could be a fraction that exhibits autocrine TGF α -EGFR signaling. The correlation of ADAM-17, TGF α and EGFR with poor prognosis implicates some role for this signaling system in metastasis. At this time, functional testing of TGF α in basal breast cancer models has not been completed. One major question that remains is: does TGF α participate in autocrine tumor cell signaling or paracrine tumor-stroma interactions. Future studies will identify which prometastatic cellular behaviors are activated by TGF α -EGFR signaling, providing insight into whether receptor-targeted therapeutics might provide benefit in a metastasis prevention paradigm.

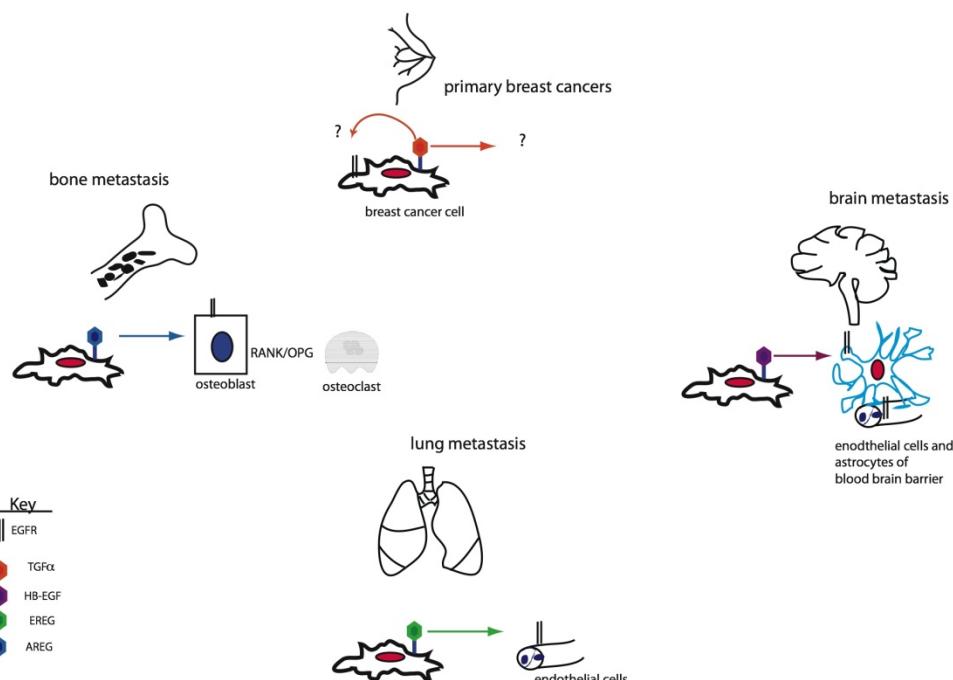


Fig. 3. Summary of EGFR-ligand signaling in models of basal breast cancer metastases. The specific ligand involved in primary tumors and metastatic site are indicated by the colored hexagons. The cell type that responds to the signal is also indicated. Paracrine signaling interactions appear to be important to metastases, whereas the relative role of autocrine versus paracrine signaling has not been explored in primary basal breast cancer.

3.2 ErbB signaling in breast cancer metastasis

Metastasis requires a set of cellular behaviors that are distinct from primary tumor formation. To spread from the site of the primary tumor to distant organs cancer cells must:

1) move out of the primary tumor and invade through local connective tissue; 2) enter capillaries (intravasation); 3) survive in the blood stream; 4) exit the blood stream (extravasation) and invade into a new organ; 5) survive in the new organ possibly as a micro metastasis; 6) adapt to the new organ and grow as a macrometastasis (virulence) (118, 119). These steps can be viewed as two generalized processes: 1) invasion, which is movement of cancer cells through normal tissue and entry into the blood stream, and 2) colonization, which is escape from the blood stream and growth in a distant organ. Substantial progress is being made in identifying metastasis genes that mediate these generalized steps of the process. Metastasis genes are thought dispensable for primary tumor initiation and growth, but are crucial to the novel processes involved in the spread of cancer (119). In general, genes that facilitate invasion for carcinomas such as breast cancer are associated with EMT. Some of the genes involved in invasion are expressed in primary tumors. Colonization is considered to be the most inefficient part of the metastatic process and the growth of a cancer cells in a novel organ is likely to require novel changes in gene expression. Thus, the expression of colonization/virulence-associated metastasis genes are thought to be limited tumors within the specific target organ, rather than being present in most primary tumors (118, 119). Due to less availability, comprehensive gene expression studies of metastases have lagged behind the studies of primary tumors. As a result, alternative strategies for identifying gene signatures that are functionally involved with metastasis have been developed. For breast cancer metastases, Massague and colleagues selected *in vitro* various subclones of the aggressive breast cancer cell line MDA-MB-231. They found that many of the subclones had differing capacities to colonize various organs after intracardiac injection into mice (12, 119-123). Gene expression profiles were generated from the subclones that colonized specific organs, and these were compared to signatures from subclones that colonized the other organs. These signatures were then compared to larger data bases generated from human primary tumors that had ultimately metastasized to the organ of interest producing a refined signature. Subsequent functional analysis of these gene signatures has resulted in the identification of specific EGFR ligands as breast cancer metastasis genes.

3.2.1 EREG and lung metastases

The identification of a lung metastatic signature was derived from subclones of the MDA-MB-231 (LM) breast cancer cells that produced lesions in the lung after intracardiac injection. Among the genes that emerged from this analysis was the ErbB ligand, EREG (121, 123). Knockdown of EREG alone failed to slow the growth of LM as primary tumors in the mammary fat pad or in lung. However, knockdown of EREG in conjunction with cyclooxygenase 2 (COX2), MMP1 and MMP2 (these genes had also been identified as upregulated in the LM signature) had a dramatic impact on both primary tumor growth and subsequent metastasis to lungs. The decreased primary tumor growth in the cells with reduced levels of EREG, COX2, MMP1 and MMP2 appeared to result from reduced angiogenesis. The LM cells recruited abundant dilated tortuous and leaky blood vessels, and the repression of these 4-genes resulted in reduced capillary branching, length and dye effusion. However, VEGF levels were not reduced in the quadruple knockdown tumors relative to controls. Also, no differences were observed in pericyte recruitment to the capillaries between the LM cells and the quadruple knockdowns, suggesting that capillary defects were the result of altered endothelial cell behavior. Also, these multiple knockdowns

exhibited reduced ability to colonize lungs after intravenous injection as compared to the parental LM cells. Close evaluation of the lungs of animals injected with the quadruple knockdown cells found abundant cells trapped in the vasculature, suggesting a failure in extravasation. The deficiencies in quadruple knockdown extravasation were also observed with an *in vitro* assay. Consistent with the knockdown studies, single therapeutic agents that targeted EGFR (such as cetuximab, an EGFR blocking antibody), COX2 (celecoxib) or MMPs (GM6001) also had modest impact on behavior of the LM cells grown as primary tumor and their spontaneous metastases to the lung. However, combinations of two agents slowed the growth of primary tumors and reduced subsequent lung colonization. The efficacy of cetuximab suggests that EREG is stimulating this the EGFR and not ErbB4. The fact that LM cells lacked upregulation of VEGF production, (an EGFR regulatory target in most cancer cells) was interpreted as evidence that EREG functioned in paracrine signaling with endothelial cells rather than being engaged in autocrine signaling.

Although microvasculature defects do not appear to be a major component of the various EGFR/- or other ErbB-knockout mice, studies of normal and tumor capillaries *in vitro* suggest signaling by family members has an influence on angiogenesis. Intriguingly, ErbB receptor expression is altered in tumor capillary endothelial cells as compared to those in normal vessels. ErbB 2, 3, 4 but minimal EGFR is found in most normal endothelial cells in culture (124). In contrast, tumor endothelial cells acquire the EGFR and down regulate ErbB3 expression both *in vitro* and *in vivo*. Endothelial cells in culture also express EGFR and ErbB4 ligands HB-EGF and NRG-1 (124-126). It is thought that HB-EGF signaling through both the EGFR and Erbb4 helps recruit pericytes to capillaries to stabilize the structures (125). The addition of exogenous EGF to tumor endothelial cells *in vitro* increases their proliferation (124). Shedding of HB-EGF and activation of the EGFR leads to down regulation of tight junction proteins and migration of normal endothelial cells (127). Also, NRG-1-ErbB4 signaling stimulates endothelial cell proliferation, migration and angiogenesis in animals; however, NRG-1 inhibits proliferation of tumor derived endothelial cells (124, 126). In light of the impact of EGFR signaling on tumor endothelial cells, breast cancer cell upregulation of EREG would contribute to the recruitment of a leaky vasculature that is common to aggressive tumors. In addition, EREG-EGFR/ErbB4 signaling might contribute to intrasavation and extrasavation by down regulation adhesion molecules between endothelial cells within capillaries. The question that remains is whether the low affinity ligand EREG might be more efficient at stimulating proliferation, migration and downregulation of junctional complexes than the high affinity ligand, HB-EGF normally present in endothelial cells.

3.2.2 HB-EGF and brain metastasis

A brain metastasis signature was derived using methods similar to those described for lung metastasis (120, 128). Among the genes that emerged as upregulated in brain metastatic cells was the ErbB ligand, HB-EGF (120). Treating mice cardiac-injected with brain seeking sublines MDA-MB-231 and CN34-BrM2C with cetuximab resulted in reduced numbers of brain metastases. Knockdown of both EREG and HB-EGF, or cetuximab treatment, reduced migration of MDA-MB-231 and CN34-BrM2C through consecutive monolayers of endothelial cells and astrocytes in a model of blood brain barrier extravasation. Brain endothelial cells and astrocytes both express the EGFR (100, 127). Activation of the EGFR has been shown to down regulate components of tight junctions in brain endothelial cells (127). This coupled with EGFR signaling induced upregulation of motility, chemokines and

extracellular matrix remodeling genes in astrocytes would likely aid in extravasation of breast cancer cells through the blood brain barrier into the parenchyma. Beyond this EGFR signaling produces reactive astrocytes that are components of the microenvironment of brain metastases. This raises the question of whether breast cancer generated EGFR ligands could play a role in breast cancer cell virulence in the brain by generating increased numbers of reactive astrocytes (129). HB-EGF is the EGFR ligand most abundantly expressed in the brain and endothelial cells, suggesting it may be the most well suited agonist to mediate in paracrine interactions among cancer cells, capillaries and the brain parenchyma.

3.2.3 AREG and bone metastasis

Tumor cell colonization of bone may be a less complex process than that of lung and brain because the capillaries in bone called sinusoids, have large openings in them to facilitate entrance of bone marrow derivatives into the circulation (119). It is thought that cancer cells may be able to exit through these openings dispensing with the intricacies of extravasation from continuous capillaries.

The growth of breast cancer macrometastases is described as a vicious cycle (130, 131). In this cycle, breast cancer cells exploit the natural renewal process based on the paracrine interactions between the bone forming osteoblast and the bone resorbing osteoclast. In normal bone, osteoblasts regulate osteoclast numbers and activity by releasing chemokines that recruit osteoclast precursors and then differentiate and activate them with a cell surface ligand for the receptor for activation of NF κ B (RANKL). Osteoblasts also produce a soluble decoy receptor osteoprotegerin (OPG) that prevents RANKL from engaging its receptor; therefore, the level of the ratio of OPG to RANKL controls osteoclastogenesis. Once activated, osteoclasts adhere to the bone surface and secrete proteases and acid that degrades mineralized matrix. Growth factors including TGF β and IGF-1 are released from the bone matrix, and this in turn stimulates new bone matrix formation by osteoblasts. This is a regenerating system that is in balance within the local environment; however, the rate of turnover and formation can be modulated by a series of endocrine hormones including PTH and calcitonin. Within the bone marrow, breast cancer cells produce a series of cytokines and growth factors including IL-11 and PTH-related protein (PTHrP) that increase the levels of RANKL relative to OPG leading to increased osteoclastogenesis. The increased bone resorption and resulting high level of growth factors enhance the survival of breast cancer cells, and also TGF β increases gene expression of IL-11 and PTHrP, which begets more osteoclasts.

Profiling bone metastasis subclones of MDA-MB-231 cells resulted in an 11-gene signature (122). Several of the genes identified were factors like IL-11 that directly altered the RANKL/OPG ratio or connective tissue factor that enhanced osteoblast proliferation. Ectopic expression of a single gene from the profile had very little impact on the ability of modestly osteolytic 231 subclones to grow within in the bone of immunocompromised mice. However, the combination of 3 of the genes from the signature induced destructive growth in bone after intracardiac injection. Among the genes identified in the signature that produced increased osteolysis when overexpressed as part of a 3 gene cassette were the proteases, MMP1 and ADAMTS-1 (12). It was unclear what the role of these molecules would play in bone metastasis. Eventually, a careful evaluation of aggressively osteolytic MDA-MB-231 lines that were engineered to overexpress ADAMTS-1 and MMP1 were found to shed dramatically increased levels of AREG. Conditioned media from the ADAMTS-1

and MMP1 engineered MDA-MB-231 lines caused an increased RANKL/OPG ratio in primary murine bone cell cultures (12, 132). The conditioned media from the MDA-MB-231-ADAMTS-1 and MMP1 cells activated osteoclastogenesis in the primary bone cell cultures, and this could be inhibited by the EGFR inhibitors gefitinib or cetuximab. Remarkably, these agents (Gefitinib 100mg/kg daily or Cetuximab 100 mg/kg weekly) completely prevented the formation of osteolytic lesions by the MDA-MB-231 ADAMTS-1+MMP1 line delivered by either the intracardiac or intratibial injection method (12). These findings clearly support the notion that EGFR signaling on cells of the osteoblast is a major regulator of the RANKL/OPG ratio, but point out the requirement for appropriate protease expression to make EGFR ligands accessible to the bone microenvironment. These experiments provide an explanation of how bone resorption could be uncoupled from bone formation by breast cancer cells because stimulation of the EGFR should block osteoblast differentiation and matrix production.

To some extent, the identification of a role for AREG in bone metastasis is confounding given that its expression is associated with ER α + breast cancers that generally have good prognosis (115). The MDA-MB-231 cells are ER α - and have a phenotype that is similar to basal cancers. So we interrogated the breast cancer transcriptome datasets GSE2034(133), GSE2603 (123), and GSE12276 (120) from the NCBI Gene Expression Omnibus to evaluate AREG expression in ER α - tumors. We found that AREG expression was lower in the ER α -negative tumors that ultimately metastasized to bone as compared to those that did not (134). A similar pattern of expression has been reported for the classical bone metastasis virulence factor PTHrP. Low expression of the peptide is observed in the primary tumors that ultimately metastasize to bone (135-137). PTHrP gene expression is thought to be activated by TGF β when breast cancer cells enter the bone microenvironment (138). In contrast to PTHrP, the enhanced activity or expression of the proteases that cleave AREG would be sufficient to increase its expression in the bone microenvironment without activating gene expression. Consistent with this concept, high expression ADAMTS-1 and MMP1 protein have been observed in primary breast cancer tumors that ultimately metastasized to bone (12). Thus, the complex post-genomic regulation of EGFR ligand processing and receptor interactions provides mechanism beyond transcription where the amplitude of signaling of this system can be increased to contribute to colonization. Given that AREG appears to be the physiological mediator of EGFR signaling in the bone, it may be ideally suited to uncouple bone formation from bone resorption, which is a component of osteolytic predominant bone metastases that arise from breast cancers.

3.3 Conclusions: EGFR ligand function in breast cancer

EGFR ligands and receptors are frequently expressed together on epithelial cells and the cancers that are derived from these tissues, including the breast. Addition of exogenous EGFR ligands to breast epithelial or cancer cells typically has a profound impact on proliferation or migratory behavior, leading to the concept that autocrine receptor signaling would contribute to tumor progression. Despite this perspective, the unraveling of the developmental breast, heart and brain phenotypes of the various family member KOs suggest that EGFR, ligand and ADAM-17 function as part of a complex paracrine-signaling network. In addition, *in vitro* and *in vivo* models based on MDA-MB-231 cells where EGFR ligands have been found to function as metastasis genes, suggest they signal in a paracrine fashion to key elements of the microenvironment. On the basis of these conclusions, we

speculate that TGF α expression in primary basal breast cancers may also be engaged in paracrine signaling with cells in the microenvironment. This conclusion suggests that the efficacy of EGFR targeted therapeutics will depend upon their uses in combination with other compounds that target the tumor microenvironment in primary basal tumors, as well as those that have metastasized to the lung, brain and bone.

4. References

- [1] Llombart-Cussac A. Improving decision-making in early breast cancer: who to treat and how? *Breast Cancer Res Treat* 2008 Dec;112 Suppl 1:15-24.
- [2] Brufsky A. Trastuzumab-based therapy for patients with HER2-positive breast cancer: from early scientific development to foundation of care. *Am J Clin Oncol* 2010 Apr;33(2):186-95.
- [3] Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001 Feb;2(2):127-37.
- [4] Wilson KJ, Gilmore JL, Foley J, Lemmon MA, Riese DJ, 2nd. Functional selectivity of EGF family peptide growth factors: implications for cancer. *Pharmacol Ther* 2009 Apr;122(1):1-8.
- [5] Daub H, Weiss FU, Wallasch C, Ullrich A. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* 1996 Feb 8;379(6565):557-60.
- [6] Iwamoto R, Mekada E. Heparin-binding EGF-like growth factor: a juxtacrine growth factor. *Cytokine Growth Factor Rev* 2000 Dec;11(4):335-44.
- [7] Prince RN, Schreiter ER, Zou P, Wiley HS, Ting AY, Lee RT, Lauffenburger DA. The heparin-binding domain of HB-EGF mediates localization to sites of cell-cell contact and prevents HB-EGF proteolytic release. *J Cell Sci* 2010 Jul 1;123(Pt 13):2308-18.
- [8] Sahin U, Weskamp G, Kelly K, Zhou HM, Higashiyama S, Peschon J, Hartmann D, Saftig P, Blobel CP. Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J Cell Biol* 2004 Mar 1;164(5):769-79.
- [9] Toussain T, Jorissen E, Reiss K, Hartmann D. (Make) stick and cut loose--disintegrin metalloproteases in development and disease. *Birth Defects Res C Embryo Today* 2006 Mar;78(1):24-46.
- [10] Zhang Q, Thomas SM, Lui VW, Xi S, Siegfried JM, Fan H, Smithgall TE, Mills GB, Grandis JR. Phosphorylation of TNF-alpha converting enzyme by gastrin-releasing peptide induces amphiregulin release and EGF receptor activation. *Proc Natl Acad Sci U S A* 2006 May 2;103(18):6901-6.
- [11] Wagstaff L, Kelwick R, Decock J, Edwards DR. The roles of ADAMTS metalloproteinases in tumorigenesis and metastasis. *Front Biosci* 2011;16:1861-72.
- [12] Lu X, Wang Q, Hu G, Van Poznak C, Fleisher M, Reiss M, Massague J, Kang Y. ADAMTS1 and MMP1 proteolytically engage EGF-like ligands in an osteolytic signaling cascade for bone metastasis. *Genes Dev* 2009 Aug 15;23(16):1882-94.
- [13] Chokki M, Eguchi H, Hamamura I, Mitsuhashi H, Kamimura T. Human airway trypsin-like protease induces amphiregulin release through a mechanism involving protease-activated receptor-2-mediated ERK activation and TNF alpha-converting enzyme activity in airway epithelial cells. *Febs J* 2005 Dec;272(24):6387-99.

- [14] Pawlowski V, Revillion F, Hebbar M, Hornez L, Peyrat JP. Prognostic value of the type I growth factor receptors in a large series of human primary breast cancers quantified with a real-time reverse transcription-polymerase chain reaction assay. *Clin Cancer Res* 2000 Nov;6(11):4217-25.
- [15] McIntyre E, Blackburn E, Brown PJ, Johnson CG, Gullick WJ. The complete family of epidermal growth factor receptors and their ligands are co-ordinately expressed in breast cancer. *Breast Cancer Res Treat* 2010 Jul;122(1):105-10.
- [16] Revillion F, Lhotellier V, Hornez L, Bonneterre J, Peyrat JP. ErbB/HER ligands in human breast cancer, and relationships with their receptors, the bio-pathological features and prognosis. *Ann Oncol* 2008 Jan;19(1):73-80.
- [17] Edwin F, Wiepz GJ, Singh R, Peet CR, Chaturvedi D, Bertics PJ, Patel TB. A historical perspective of the EGF receptor and related systems. *Methods Mol Biol* 2006;327:1-24.
- [18] Riese DJ, Stern DF. Specificity within the EGF family/ErbB receptor family signaling network. *Bioessays* 1998 Jan;20(1):41-8.
- [19] Lemmon MA. Ligand-induced ErbB receptor dimerization. *Exp Cell Res* 2009 Feb 15;315(4):638-48.
- [20] Riese DJ, Gallo RM, Settleman J. Mutational activation of ErbB family receptor tyrosine kinases: insights into mechanisms of signal transduction and tumorigenesis. *Bioessays* 2007 Jun;29(6):558-65.
- [21] Schulze WX, Deng L, Mann M. Phosphotyrosine interactome of the ErbB-receptor kinase family. *Mol Syst Biol* 2005;1:2005 0008.
- [22] Roepstorff K, Grandal MV, Henriksen L, Knudsen SL, Lerdrup M, Grovdal L, Willumsen BM, van Deurs B. Differential effects of EGFR ligands on endocytic sorting of the receptor. *Traffic* 2009 Aug;10(8):1115-27.
- [23] Avraham R, Yarden Y. Feedback regulation of EGFR signalling: decision making by early and delayed loops. *Nat Rev Mol Cell Biol* 2011 Feb;12(2):104-17.
- [24] Bublil EM, Yarden Y. The EGF receptor family: spearheading a merger of signaling and therapeutics. *Curr Opin Cell Biol* 2007 Apr;19(2):124-34.
- [25] Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. *Nat Rev Mol Cell Biol* 2006 Jul;7(7):505-16.
- [26] Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987 Jan 9;235(4785):177-82.
- [27] Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 1995 Jul;19(3):183-232.
- [28] Earp HS, Dawson TL, Li X, Yu H. Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research. *Breast Cancer Res Treat* 1995 Jul;35(1):115-32.
- [29] Wada T, Qian XL, Greene MI. Intermolecular association of the p185neu protein and EGF receptor modulates EGF receptor function. *Cell* 1990 Jun 29;61(7):1339-47.

- [30] Wang Z, Zhang L, Yeung TK, Chen X. Endocytosis deficiency of epidermal growth factor (EGF) receptor-ErbB2 heterodimers in response to EGF stimulation. *Mol Biol Cell*1999 May;10(5):1621-36.
- [31] Hendriks BS, Wiley HS, Lauffenburger D. HER2-mediated effects on EGFR endosomal sorting: analysis of biophysical mechanisms. *Biophys J*2003 Oct;85(4):2732-45.
- [32] Jones JT, Akita RW, Sliwkowski MX. Binding specificities and affinities of egf domains for ErbB receptors. *FEBS Lett*1999 Mar 26;447(2-3):227-31.
- [33] Liu W, Volpe MA, Zscheppang K, Nielsen HC, Dammann CE. ErbB4 regulates surfactant synthesis and proliferation in adult rat pulmonary epithelial cells. *Exp Lung Res*2009 Feb;35(1):29-47.
- [34] Zscheppang K, Korenbaum E, Bueter W, Ramadurai SM, Nielsen HC, Dammann CE. ErbB receptor dimerization, localization, and co-localization in mouse lung type II epithelial cells. *Pediatr Pulmonol*2006 Dec;41(12):1205-12.
- [35] Hatakeyama M, Yumoto N, Yu X, Shirouzu M, Yokoyama S, Konagaya A. Transformation potency of ErbB heterodimer signaling is determined by B-Raf kinase. *Oncogene*2004 Jun 24;23(29):5023-31.
- [36] Frolov A, Schuller K, Tzeng CW, Cannon EE, Ku BC, Howard JH, Vickers SM, Heslin MJ, Buchsbaum DJ, Arnoletti JP. ErbB3 expression and dimerization with EGFR influence pancreatic cancer cell sensitivity to erlotinib. *Cancer Biol Ther*2007 Apr;6(4):548-54.
- [37] Liles JS, Arnoletti JP, Tzeng CW, Howard JH, Kossenkov AV, Kulesza P, Heslin MJ, Frolov A. ErbB3 expression promotes tumorigenesis in pancreatic adenocarcinoma. *Cancer Biol Ther*2010 Sep;10(6):555-63.
- [38] Waterman H, Sabanai I, Geiger B, Yarden Y. Alternative intracellular routing of ErbB receptors may determine signaling potency. *J Biol Chem*1998 May 29;273(22):13819-27.
- [39] French AR, Sudlow GP, Wiley HS, Lauffenburger DA. Postendocytic trafficking of epidermal growth factor-receptor complexes is mediated through saturable and specific endosomal interactions. *J Biol Chem*1994 Jun 3;269(22):15749-55.
- [40] Reddy CC, Niyogi SK, Wells A, Wiley HS, Lauffenburger DA. Engineering epidermal growth factor for enhanced mitogenic potency. *Nat Biotechnol*1996 Dec;14(13):1696-9.
- [41] Willmarth NE, Ethier SP. Autocrine and juxtacrine effects of amphiregulin on the proliferative, invasive, and migratory properties of normal and neoplastic human mammary epithelial cells. *J Biol Chem*2006 Dec 8;281(49):37728-37.
- [42] Streicher KL, Willmarth NE, Garcia J, Boerner JL, Dewey TG, Ethier SP. Activation of a nuclear factor kappaB/interleukin-1 positive feedback loop by amphiregulin in human breast cancer cells. *Mol Cancer Res*2007 Aug;5(8):847-61.
- [43] Willmarth NE, Baillo A, Dziubinski ML, Wilson K, Riese DJ, 2nd, Ethier SP. Altered EGFR localization and degradation in human breast cancer cells with an amphiregulin/EGFR autocrine loop. *Cell Signal*2009 Feb;21(2):212-9.
- [44] Shoyab M, McDonald VL, Bradley JG, Todaro GJ. Amphiregulin: a bifunctional growth-modulating glycoprotein produced by the phorbol 12-myristate 13-acetate-

- treated human breast adenocarcinoma cell line MCF-7. Proc Natl Acad Sci U S A1988 Sep;85(17):6528-32.
- [45] Shoyab M, Plowman GD, McDonald VL, Bradley JG, Todaro GJ. Structure and function of human amphiregulin: a member of the epidermal growth factor family. Science1989 Feb 24;243(4894 Pt 1):1074-6.
- [46] Stoll SW, Johnson JL, Bhasin A, Johnston A, Gudjonsson JE, Rittie L, Elder JT. Metalloproteinase-mediated, context-dependent function of amphiregulin and HB-EGF in human keratinocytes and skin. J Invest Dermatol2010 Jan;130(1):295-304.
- [47] Barnard JA, Graves-Deal R, Pittelkow MR, DuBois R, Cook P, Ramsey GW, Bishop PR, Damstrup L, Coffey RJ. Auto- and cross-induction within the mammalian epidermal growth factor-related peptide family. J Biol Chem1994 Sep 9;269(36):22817-22.
- [48] Cook PW, Mattox PA, Keeble WW, Pittelkow MR, Plowman GD, Shoyab M, Adelman JP, Shipley GD. A heparin sulfate-regulated human keratinocyte autocrine factor is similar or identical to amphiregulin. Mol Cell Biol1991 May;11(5):2547-57.
- [49] Neelam B, Richter A, Chamberlin SG, Puddicombe SM, Wood L, Murray MB, Nandagopal K, Niyogi SK, Davies DE. Structure-function studies of ligand-induced epidermal growth factor receptor dimerization. Biochemistry1998 Apr 7;37(14):4884-91.
- [50] Adam R, Drummond DR, Solic N, Holt SJ, Sharma RP, Chamberlin SG, Davies DE. Modulation of the receptor binding affinity of amphiregulin by modification of its carboxyl terminal tail. Biochim Biophys Acta1995 Apr 6;1266(1):83-90.
- [51] Gilmore JL, Scott JA, Bouizar Z, Robling A, Pitfield SE, Riese DJ, 2nd, Foley J. Amphiregulin-EGFR signaling regulates PTHrP gene expression in breast cancer cells. Breast Cancer Res Treat2007 Sep 20.
- [52] Stern KA, Place TL, Lill NL. EGF and amphiregulin differentially regulate Cbl recruitment to endosomes and EGF receptor fate. Biochem J2008 Mar 15;410(3):585-94.
- [53] Chung E, Graves-Deal R, Franklin JL, Coffey RJ. Differential effects of amphiregulin and TGF-alpha on the morphology of MDCK cells. Exp Cell Res2005 Sep 10;309(1):149-60.
- [54] Kochupurakkal BS, Harari D, Di-Segni A, Maik-Rachline G, Lyass L, Gur G, Kerber G, Citri A, Lavi S, Eilam R, Chalifa-Caspi V, Eshhar Z, Pikarsky E, Pinkas-Kramarski R, Bacus SS, Yarden Y. Epigen, the last ligand of ErbB receptors, reveals intricate relationships between affinity and mitogenicity. J Biol Chem2005 Mar 4;280(9):8503-12.
- [55] Strachan L, Murison JG, Prestidge RL, Sleeman MA, Watson JD, Kumble KD. Cloning and biological activity of epigen, a novel member of the epidermal growth factor superfamily. J Biol Chem2001 May 25;276(21):18265-71.
- [56] Elenius K, Paul S, Allison G, Sun J, Klagsbrun M. Activation of HER4 by heparin-binding EGF-like growth factor stimulates chemotaxis but not proliferation. Embo J1997 Mar 17;16(6):1268-78.

- [57] Higashiyama S, Abraham JA, Miller J, Fiddes JC, Klagsbrun M. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science*1991 Feb 22;251(4996):936-9.
- [58] Nishi E, Klagsbrun M. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a mediator of multiple physiological and pathological pathways. *Growth Factors*2004 Dec;22(4):253-60.
- [59] Shing Y, Christofori G, Hanahan D, Ono Y, Sasada R, Igarashi K, Folkman J. Betacellulin: a mitogen from pancreatic beta cell tumors. *Science*1993 Mar 12;259(5101):1604-7.
- [60] Sweeney C, Lai C, Riese DJ, 2nd, Diamanti AJ, Cantley LC, Carraway KL, 3rd. Ligand discrimination in signaling through an ErbB4 receptor homodimer. *J Biol Chem*2000 Jun 30;275(26):19803-7.
- [61] Tada H, Sasada R, Kawaguchi Y, Kojima I, Gullick WJ, Salomon DS, Igarashi K, Seno M, Yamada H. Processing and juxtameric activity of membrane-anchored betacellulin. *J Cell Biochem*1999 Mar 1;72(3):423-34.
- [62] Komurasaki T, Toyoda H, Uchida D, Morimoto S. Epiregulin binds to epidermal growth factor receptor and ErbB-4 and induces tyrosine phosphorylation of epidermal growth factor receptor, ErbB-2, ErbB-3 and ErbB-4. *Oncogene*1997 Dec 4;15(23):2841-8.
- [63] Sasaki E, Arakawa T, Fujiwara Y, Kawada N, Fukuda T, Higuchi K, Komurasaki T, Kobayashi K. Epiregulin stimulates proliferation of rabbit gastric cells in primary culture through autophosphorylation of the epidermal growth factor receptor. *Eur J Pharmacol*1997 Nov 12;338(3):253-8.
- [64] Riese DJ, 2nd, Komurasaki T, Plowman GD, Stern DF. Activation of ErbB4 by the bifunctional epidermal growth factor family hormone epiregulin is regulated by ErbB2. *J Biol Chem*1998 May 1;273(18):11288-94.
- [65] Shelly M, Pinkas-Kramarski R, Guarino BC, Waterman H, Wang LM, Lyass L, Alimandi M, Kuo A, Bacus SS, Pierce JH, Andrews GC, Yarden Y. Epiregulin is a potent pan-ErbB ligand that preferentially activates heterodimeric receptor complexes. *J Biol Chem*1998 Apr 24;273(17):10496-505.
- [66] Cardiff RD, Wellings SR. The comparative pathology of human and mouse mammary glands. *J Mammary Gland Biol Neoplasia*1999 Jan;4(1):105-22.
- [67] Hens JR, Wysolmerski JJ. Key stages of mammary gland development: molecular mechanisms involved in the formation of the embryonic mammary gland. *Breast Cancer Res*2005;7(5):220-4.
- [68] Fendrick JL, Raafat AM, Haslam SZ. Mammary gland growth and development from the postnatal period to postmenopause: ovarian steroid receptor ontogeny and regulation in the mouse. *J Mammary Gland Biol Neoplasia*1998 Jan;3(1):7-22.
- [69] Nandi S. Endocrine control of mammary gland development and function in the C3H/He Crgl mouse. *J Natl Cancer Inst*1958 Dec;21(6):1039-63.
- [70] Snedeker SM, Brown CF, DiAugustine RP. Expression and functional properties of transforming growth factor alpha and epidermal growth factor during mouse mammary gland ductal morphogenesis. *Proc Natl Acad Sci U S A*1991 Jan 1;88(1):276-80.

- [71] Watson CJ, Khaled WT. Mammary development in the embryo and adult: a journey of morphogenesis and commitment. *Development* 2008 Mar;135(6):995-1003.
- [72] Brisken C, Kaur S, Chavarria TE, Binart N, Sutherland RL, Weinberg RA, Kelly PA, Ormandy CJ. Prolactin controls mammary gland development via direct and indirect mechanisms. *Dev Biol* 1999 Jun 1;210(1):96-106.
- [73] Brisken C, Park S, Vass T, Lydon JP, O'Malley BW, Weinberg RA. A paracrine role for the epithelial progesterone receptor in mammary gland development. *Proc Natl Acad Sci U S A* 1998 Apr 28;95(9):5076-81.
- [74] Schroeder JA, Lee DC. Dynamic expression and activation of ERBB receptors in the developing mouse mammary gland. *Cell Growth Differ* 1998 Jun;9(6):451-64.
- [75] Sebastian J, Richards RG, Walker MP, Wiesen JF, Werb Z, Derynck R, Hom YK, Cunha GR, DiAugustine RP. Activation and function of the epidermal growth factor receptor and erbB-2 during mammary gland morphogenesis. *Cell Growth Differ* 1998 Sep;9(9):777-85.
- [76] Wiesen JF, Young P, Werb Z, Cunha GR. Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development. *Development* 1999 Jan;126(2):335-44.
- [77] Kenney NJ, Huang RP, Johnson GR, Wu JX, Okamura D, Matheny W, Kordon E, Gullick WJ, Plowman G, Smith GH, et al. Detection and location of amphiregulin and Cripto-1 expression in the developing postnatal mouse mammary gland. *Mol Reprod Dev* 1995 Jul;41(3):277-86.
- [78] Luetteke NC, Qiu TH, Fenton SE, Troyer KL, Riedel RF, Chang A, Lee DC. Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development* 1999 Jun;126(12):2739-50.
- [79] Ciarloni L, Mallepell S, Brisken C. Amphiregulin is an essential mediator of estrogen receptor alpha function in mammary gland development. *Proc Natl Acad Sci U S A* 2007 Mar 27;104(13):5455-60.
- [80] Rho JY, Wada-Kiyama Y, Onishi Y, Kiyama R, Sakuma Y. Expressional regulation of neuronal and cancer-related genes by estrogen in adult female rats. *Endocr Res* 2004 May;30(2):257-67.
- [81] Britton DJ, Hutcheson IR, Knowlden JM, Barrow D, Giles M, McClelland RA, Gee JM, Nicholson RI. Bidirectional cross talk between ERalpha and EGFR signalling pathways regulates tamoxifen-resistant growth. *Breast Cancer Res Treat* 2006 Mar;96(2):131-46.
- [82] Sternlicht MD, Sunnarborg SW, Kouros-Mehr H, Yu Y, Lee DC, Werb Z. Mammary ductal morphogenesis requires paracrine activation of stromal EGFR via ADAM17-dependent shedding of epithelial amphiregulin. *Development* 2005 Sep;132(17):3923-33.
- [83] Asselin-Labat ML, Vaillant F, Shackleton M, Bouras T, Lindeman GJ, Visvader JE. Delineating the epithelial hierarchy in the mouse mammary gland. *Cold Spring Harb Symp Quant Biol* 2008;73:469-78.

- [84] Eirew P, Stingl J, Raouf A, Turashvili G, Aparicio S, Emerman JT, Eaves CJ. A method for quantifying normal human mammary epithelial stem cells with in vivo regenerative ability. *Nat Med*2008 Dec;14(12):1384-9.
- [85] Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH, Asselin-Labat ML, Gyorki DE, Ward T, Partanen A, Feleppa F, Huschtscha LI, Thorne HJ, Fox SB, Yan M, French JD, Brown MA, Smyth GK, Visvader JE, Lindeman GJ. Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. *Nat Med*2009 Aug;15(8):907-13.
- [86] Asselin-Labat ML, Sutherland KD, Barker H, Thomas R, Shackleton M, Forrest NC, Hartley L, Robb L, Grosveld FG, van der Wees J, Lindeman GJ, Visvader JE. Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. *Nat Cell Biol*2007 Feb;9(2):201-9.
- [87] Jackson-Fisher AJ, Bellinger G, Breindel JL, Tavassoli FA, Booth CJ, Duong JK, Stern DF. ErbB3 is required for ductal morphogenesis in the mouse mammary gland. *Breast Cancer Res*2008;10(6):R96.
- [88] Jackson-Fisher AJ, Bellinger G, Ramabhadran R, Morris JK, Lee KF, Stern DF. ErbB2 is required for ductal morphogenesis of the mammary gland. *Proc Natl Acad Sci U S A*2004 Dec 7;101(49):17138-43.
- [89] Tidcombe H, Jackson-Fisher A, Mathers K, Stern DF, Gassmann M, Golding JP. Neural and mammary gland defects in ErbB4 knockout mice genetically rescued from embryonic lethality. *Proc Natl Acad Sci U S A*2003 Jul 8;100(14):8281-6.
- [90] Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C. Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature*1995 Nov 23;378(6555):394-8.
- [91] Erickson SL, O'Shea KS, Ghaboosi N, Loverro L, Frantz G, Bauer M, Lu LH, Moore MW. ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice. *Development*1997 Dec;124(24):4999-5011.
- [92] Kramer R, Bucay N, Kane DJ, Martin LE, Tarpley JE, Theill LE. Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. *Proc Natl Acad Sci U S A*1996 May 14;93(10):4833-8.
- [93] Gassmann M, Casagrande F, Orioli D, Simon H, Lai C, Klein R, Lemke G. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature*1995 Nov 23;378(6555):390-4.
- [94] Sanchez-Soria P, Camenisch TD. ErbB signaling in cardiac development and disease. *Semin Cell Dev Biol*2010 Dec;21(9):929-35.
- [95] Schroeder JA, Jackson LF, Lee DC, Camenisch TD. Form and function of developing heart valves: coordination by extracellular matrix and growth factor signaling. *J Mol Med*2003 Jul;81(7):392-403.
- [96] Sibilia M, Wagner EF. Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science*1995 Jul 14;269(5221):234-8.
- [97] Jackson LF, Qiu TH, Sunnarborg SW, Chang A, Zhang C, Patterson C, Lee DC. Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. *Embo J*2003 Jun 2;22(11):2704-16.

- [98] Kornblum HI, Hussain R, Wiesen J, Miettinen P, Zurcher SD, Chow K, Deryck R, Werb Z. Abnormal astrocyte development and neuronal death in mice lacking the epidermal growth factor receptor. *J Neurosci Res* 1998 Sep 15;33(6):697-717.
- [99] Kornblum HI, Zurcher SD, Werb Z, Deryck R, Seroogy KB. Multiple trophic actions of heparin-binding epidermal growth factor (HB-EGF) in the central nervous system. *Eur J Neurosci* 1999 Sep;11(9):3236-46.
- [100] Kornblum HI, Hussain RJ, Bronstein JM, Gall CM, Lee DC, Seroogy KB. Prenatal ontogeny of the epidermal growth factor receptor and its ligand, transforming growth factor alpha, in the rat brain. *J Comp Neurol* 1997 Apr 7;380(2):243-61.
- [101] Liu B, Neufeld AH. Activation of epidermal growth factor receptors in astrocytes: from development to neural injury. *J Neurosci Res* 2007 Dec;85(16):3523-9.
- [102] Kang W, Hebert JM. Signaling pathways in reactive astrocytes, a genetic perspective. *Mol Neurobiol* 2011 Jun;43(3):147-54.
- [103] Ibbotson KJ, D'Souza SM, Ng KW, Osborne CK, Niall M, Martin TJ, Mundy GR. Tumor-derived growth factor increases bone resorption in a tumor associated with humoral hypercalcemia of malignancy. *Science* 1983 Sep 23;221(4617):1292-4.
- [104] Ibbotson KJ, D'Souza SM, Smith DD, Carpenter G, Mundy GR. EGF receptor antiserum inhibits bone resorbing activity produced by a rat Leydig cell tumor associated with the humoral hypercalcemia of malignancy. *Endocrinology* 1985 Jan;116(1):469-71.
- [105] Shupnik MA, Ip NY, Tashjian AH, Jr. Characterization and regulation of receptors for epidermal growth factor in mouse calvaria. *Endocrinology* 1980 Dec;107(6):1738-46.
- [106] Sibilia M, Wagner B, Hoebertz A, Elliott C, Marino S, Jochum W, Wagner EF. Mice humanised for the EGF receptor display hypomorphic phenotypes in skin, bone and heart. *Development* 2003 Oct;130(19):4515-25.
- [107] Qin L, Partridge NC. Stimulation of amphiregulin expression in osteoblastic cells by parathyroid hormone requires the protein kinase A and cAMP response element-binding protein signaling pathway. *J Cell Biochem* 2005 Oct 15;96(3):632-40.
- [108] Qin L, Qiu P, Wang L, Li X, Swarthout JT, Soteropoulos P, Tolias P, Partridge NC. Gene expression profiles and transcription factors involved in parathyroid hormone signaling in osteoblasts revealed by microarray and bioinformatics. *J Biol Chem* 2003 May 30;278(22):19723-31.
- [109] Cole JA. Parathyroid hormone activates mitogen-activated protein kinase in opossum kidney cells. *Endocrinology* 1999 Dec;140(12):5771-9.
- [110] van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002 Jan 31;415(6871):530-6.
- [111] van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van der Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, Bernards R. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002 Dec 19;347(25):1999-2009.

- [112] Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lonning P, Borresen-Dale AL. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 2001 Sep 11;98(19):10869-74.
- [113] Wiseman SM, Makretsov N, Nielsen TO, Gilks B, Yorida E, Cheang M, Turbin D, Gelmon K, Huntsman DG. Coexpression of the type 1 growth factor receptor family members HER-1, HER-2, and HER-3 has a synergistic negative prognostic effect on breast carcinoma survival. *Cancer* 2005 May 1;103(9):1770-7.
- [114] Zhu Y, Sullivan LL, Nair SS, Williams CC, Pandey AK, Marrero L, Vadlamudi RK, Jones FE. Coregulation of estrogen receptor by ERBB4/HER4 establishes a growth-promoting autocrine signal in breast tumor cells. *Cancer Res* 2006 Aug 15;66(16):7991-8.
- [115] McGowan PM, McKiernan E, Bolster F, Ryan BM, Hill AD, McDermott EW, Evoy D, O'Higgins N, Crown J, Duffy MJ. ADAM-17 predicts adverse outcome in patients with breast cancer. *Ann Oncol* 2008 Jun;19(6):1075-81.
- [116] Burness ML, Grushko TA, Olopade OI. Epidermal growth factor receptor in triple-negative and basal-like breast cancer: promising clinical target or only a marker? *Cancer J* 2010 Jan-Feb;16(1):23-32.
- [117] Viale G, Rotmensz N, Maisonneuve P, Bottiglieri L, Montagna E, Luini A, Veronesi P, Intra M, Torrisi R, Cardillo A, Campagnoli E, Goldhirsch A, Colleoni M. Invasive ductal carcinoma of the breast with the "triple-negative" phenotype: prognostic implications of EGFR immunoreactivity. *Breast Cancer Res Treat* 2009 Jul;116(2):317-28.
- [118] Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. *Science* 2011 Mar 25;331(6024):1559-64.
- [119] Nguyen DX, Bos PD, Massague J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer* 2009 Apr;9(4):274-84.
- [120] Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, Nguyen DX, Minn AJ, van de Vijver MJ, Gerald WL, Foekens JA, Massague J. Genes that mediate breast cancer metastasis to the brain. *Nature* 2009 Jun 18;459(7249):1005-9.
- [121] Gupta GP, Nguyen DX, Chiang AC, Bos PD, Kim JY, Nadal C, Gomis RR, Manova-Todorova K, Massague J. Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. *Nature* 2007 Apr 12;446(7137):765-70.
- [122] Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, Guise TA, Massague J. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 2003 Jun;3(6):537-49.
- [123] Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massague J. Genes that mediate breast cancer metastasis to lung. *Nature* 2005 Jul 28;436(7050):518-24.
- [124] Amin DN, Hida K, Bielenberg DR, Klagsbrun M. Tumor endothelial cells express epidermal growth factor receptor (EGFR) but not ErbB3 and are responsive to EGF and to EGFR kinase inhibitors. *Cancer Res* 2006 Feb 15;66(4):2173-80.

- [125] Iivanainen E, Nelimarkka L, Elenius V, Heikkinen SM, Junttila TT, Sihombing L, Sundvall M, Maatta JA, Laine VJ, Yla-Herttuala S, Higashiyama S, Alitalo K, Elenius K. Angiopoietin-regulated recruitment of vascular smooth muscle cells by endothelial-derived heparin binding EGF-like growth factor. *FASEB J*2003 Sep;17(12):1609-21.
- [126] Russell KS, Stern DF, Polverini PJ, Bender JR. Neuregulin activation of ErbB receptors in vascular endothelium leads to angiogenesis. *Am J Physiol*1999 Dec;277(6 Pt 2):H2205-11.
- [127] Chen F, Hori T, Ohashi N, Baine AM, Eckman CB, Nguyen JH. Occludin is regulated by epidermal growth factor receptor activation in brain endothelial cells and brains of mice with acute liver failure. *Hepatology*2011 Apr;53(4):1294-305.
- [128] Yoneda T, Williams PJ, Hiraga T, Niewolna M, Nishimura R. A bone-seeking clone exhibits different biological properties from the MDA-MB-231 parental human breast cancer cells and a brain-seeking clone in vivo and in vitro. *J Bone Miner Res*2001 Aug;16(8):1486-95.
- [129] Fitzgerald DP, Palmieri D, Hua E, Hargrave E, Herring JM, Qian Y, Vega-Valle E, Weil RJ, Stark AM, Vortmeyer AO, Steeg PS. Reactive glia are recruited by highly proliferative brain metastases of breast cancer and promote tumor cell colonization. *Clin Exp Metastasis*2008;25(7):799-810.
- [130] Guise TA, Mohammad KS, Clines G, Stebbins EG, Wong DH, Higgins LS, Vessella R, Corey E, Padalecki S, Suva L, Chirgwin JM. Basic mechanisms responsible for osteolytic and osteoblastic bone metastases. *Clin Cancer Res*2006 Oct 15;12(20 Pt 2):6213s-6s.
- [131] Guise TA, Mundy GR. Cancer and bone. *Endocr Rev*1998 Feb;19(1):18-54.
- [132] Zhu J, Jia X, Xiao G, Kang Y, Partridge NC, Qin L. EGF-like ligands stimulate osteoclastogenesis by regulating expression of osteoclast regulatory factors by osteoblasts: implications for osteolytic bone metastases. *J Biol Chem*2007 Sep 14;282(37):26656-64.
- [133] Wang Y, Klijn JG, Zhang Y, Sieuwerts AM, Look MP, Yang F, Talantov D, Timmermans M, Meijer-van Gelder ME, Yu J, Jatkoe T, Berns EM, Atkins D, Foekens JA. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet*2005 Feb 19-25;365(9460):671-9.
- [134] Foley J, Nickerson NK, Nam S, Allen KT, Gilmore JL, Nephew KP, Riese DJ, 2nd. EGFR signaling in breast cancer: bad to the bone. *Semin Cell Dev Biol*2010 Dec;21(9):951-60.
- [135] Henderson M, Danks J, Moseley J, Slavin J, Harris T, McKinlay M, Hopper J, Martin T. Parathyroid hormone-related protein production by breast cancers, improved survival, and reduced bone metastases. *J Natl Cancer Inst*2001 Feb 7;93(3):234-7.
- [136] Powell GJ, Southby J, Danks JA, Stillwell RG, Hayman JA, Henderson MA, Bennett RC, Martin TJ. Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared with other sites. *Cancer Res*1991 Jun 1;51(11):3059-61.

- [137] Southby J, Kissin MW, Danks JA, Hayman JA, Moseley JM, Henderson MA, Bennett RC, Martin TJ. Immunohistochemical localization of parathyroid hormone-related protein in human breast cancer. *Cancer Res* 1990 Dec 1;50(23):7710-6.
- [138] Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, Massague J, Mundy GR, Guise TA. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* 1999 Jan;103(2):197-206.



Review

EGFR signaling in breast cancer: Bad to the bone

John Foley^{a,c,d,*}, Nicole K. Nickerson^a, Seungyoon Nam^a, Kah Tan Allen^a, Jennifer L. Gilmore^{a,b,d,e}, Kenneth P. Nephew^{a,d}, David J. Riese II^{b,e,**}

^a Medical Sciences Program, Indiana University School of Medicine, Bloomington, IN 47405, United States

^b Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University College of Pharmacy, West Lafayette, IN 47907, United States

^c Department of Dermatology, Indiana University School of Medicine, Indianapolis, IN 46202, United States

^d Indiana University Cancer Center, Indiana University School of Medicine, Indianapolis, IN 46202, United States

^e Purdue University Center for Cancer Research, Purdue University, West Lafayette, IN 47907, United States

ARTICLE INFO

Article history:

Available online 9 September 2010

Keywords:

Epidermal growth factor receptor
Triple-negative tumors
Resistance to endocrine therapy
Stem-like tumor cells
Bone metastasis

ABSTRACT

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases. This family includes EGFR/ErbB1/HER1, ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4. For many years it was believed that EGFR plays a minor role in the development and progression of breast malignancies. However, recent findings have led investigators to revisit these beliefs. Here we will review these findings and propose roles that EGFR may play in breast malignancies. In particular, we will discuss the potential roles that EGFR may play in triple-negative tumors, resistance to endocrine therapies, maintenance of stem-like tumor cells, and bone metastasis. Thus, we will propose the contexts in which EGFR may be a therapeutic target.

© 2010 Elsevier Ltd. All rights reserved.

Contents

1. Introduction	952
1.1. EGFR ligands and signaling	952
1.2. EGFR signaling specificity	952
2. Manuscript body	952
2.1. EGFR and primary breast tumors	952
2.1.1. Triple-negative, basal breast tumors	953
2.1.2. Resistance to antiestrogens	954
2.1.3. Breast cancer stem cells	954
2.2. EGFR and bone metastasis	954
2.2.1. Latent bone colonization by breast tumor cells	954
2.2.2. Bone metastasis: a vicious cycle	955
2.2.3. EGFR and osteolysis	956
2.2.4. EGFR and osteoblast function	956
2.2.5. EGFR and PTHrP	956
2.2.6. EGFR ligands and activating proteases as bone metastasis virulence factors	956
Acknowledgements	957
References	957

Abbreviations: ADAM, a disintegrin and metalloproteinase; ADAMTS, ADAM with thrombospondin motif; AREG, amphiregulin; CSF-1, colony-stimulating factor-1; DTC, disseminated tumor cell; EGFR, epidermal growth factor receptor; ER, estrogen receptor; OPG, osteoprotegerin; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; RANK, receptor activator of nuclear factor β -ligand; RANKL, RANK ligand; TACE, tumor necrosis factor alpha converting enzyme.

* Corresponding author at: Medical Sciences, Indiana University, Jordan Hall, Bloomington, IN 47405, United States. Tel.: +1 812 855 3189.

** Corresponding author at: Harrison School of Pharmacy, Auburn University, 2316-H Walker Building, Auburn, AL 36849, United States. Tel.: +1 334 844 8348; fax: +1 815 301 3212.

E-mail addresses: jgfoley@indiana.edu (J. Foley), driese@purdue.edu, driese@auburn.edu (D.J. Riese II).

1. Introduction

The study of breast cancer has provided opportunities to test concepts emerging from basic studies of cell proliferation, signal transduction and developmental biology. One subject of these basic studies is the epidermal growth factor receptor (EGFR) or ErbB family of receptor tyrosine kinases. This family includes EGFR/ErbB1/HER1, ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4. These receptors play distinct roles in breast malignancies [1–15]. ErbB2 is a therapeutic target in breast tumors that overexpress the receptor. In contrast, the roles that ErbB4 plays in breast malignancies remain a subject of opposing views. For many years it was believed that EGFR plays a minor role in the development and progression of breast malignancies. However, recent findings have led investigators to revisit these beliefs. Here we will review these findings and propose roles that EGFR may play in breast malignancies. Thus, we will propose the contexts in which EGFR may be a therapeutic target.

1.1. EGFR ligands and signaling

EGFR signaling is stimulated by members of the epidermal growth factor (EGF) family of peptide growth factors, whose roles in stimulating ErbB receptor signaling and coupling to biological responses have been intensively studied [2,12,16,17]. EGFR agonists include the epidermal growth factor (EGF), transforming growth factor alpha (TGF α), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AREG), epiregulin (EPI), epigen (EPG), betacellulin (BTC) and neuregulin (NRG) 2 β . These agonists are expressed as integral membrane proteins and are cleaved by metalloproteinases to release soluble, mature ligands. These metalloproteinases are typically members of the ADAM (a disintegrin and metalloproteinase) family of membrane proteases. For example, ADAM17 (tumor necrosis factor α converting enzyme – TACE) cleaves AREG, EPI, HB-EGF and TGF α [18–22]. Because cleavage of the ligand precursors is required for release of soluble, mature ligands, ligand cleavage represents a potential point in which agonist-induced EGFR signaling can be regulated. However, the transmembrane ligands stimulate EGFR signaling on adjacent cells, apparently through a juxtacrine signaling mechanism that may mediate the stromal–epithelial interactions characteristic of the breast [23–25].

The mechanisms by which EGFR signaling is stimulated by agonist binding have been extensively studied [16,17,26,27]. To summarize, EGFR consists of an extracellular domain, a hydrophobic transmembrane domain, an intracellular catalytic tyrosine kinase domain, and several intracellular tyrosine residues whose phosphorylation is responsible for coupling to downstream effectors. Ligand binding to the extracellular domain stabilizes the EGFR in an extended conformation that is competent for receptor dimerization. Dimerization then enables the cytoplasmic domain of one receptor monomer (the regulatory monomer) to stabilize the tyrosine kinase domain of another monomer (the catalytic monomer) in the active conformation and presents the tyrosine residues of the regulatory monomer to the catalytic site of the catalytic monomer. In this manner EGFR dimerization enables its tyrosine phosphorylation (Fig. 1).

Approximately 10 EGFR tyrosine residues are phosphorylated following ligand engagement and receptor dimerization [17,28]. These phosphorylation sites bind adapter proteins and other signaling molecules that possess SH2 (Src-homology domain 2) or PTB (phospho-tyrosine binding) motifs. Several of the phosphorylated tyrosine residues can bind unique effectors and each EGFR agonist is likely to stimulate EGFR phosphorylation at a unique subset of tyrosine residues. Thus, EGFR agonists typically stimulate EGFR coupling to multiple effectors, including Ras, MAPK, Src, STAT 3/5,

PLC γ , PKC, and PI3 kinase [17,29]. These effectors are typically coupled to increased survival, proliferation, motility and invasiveness displayed by malignant tumor cells.

In contrast, some EGFR agonists also stimulate coupling to downstream molecules that negatively regulate the receptor. For instance, phosphorylation of EGFR Tyr974 triggers EGFR endocytosis and phosphorylation of EGFR Tyr1045 triggers Cbl-dependent EGFR ubiquitination and proteosomal degradation [17,30]. EGFR phosphorylation also triggers EGFR binding to SHPTP protein tyrosine phosphatases, in which in turn dephosphorylate EGFR [17,31,32]. Thus, EGFR agonists also stimulate pathways that negatively regulate EGFR coupling to malignant phenotypes and the balance between these positive and negative regulators of EGFR coupling to malignant phenotypes may be altered in tumor cells.

1.2. EGFR signaling specificity

Several factors contribute to EGFR signaling specificity. One is the presence of other ErbB family receptors. For example, ErbB2 can stabilize EGFR in a conformation that is competent for dimerization and tyrosine phosphorylation even in the absence of ligand binding, thereby contributing to ligand-independent EGFR signaling and increased ligand affinity for the EGFR [16,33,34]. Furthermore, ErbB2 and ErbB4 heterodimerize with EGFR upon agonist binding to EGFR. This results in phosphorylation of the heterodimerization partner (ErbB2 or ErbB4) and may result in phosphorylation of a different set of EGFR tyrosine residues [16,33]. The latter mechanism may account for the observation that heterodimerization of ErbB2 with EGFR alters EGFR endocytosis and intracellular trafficking [35–37]. In any event, agonist-induced heterodimerization of EGFR with a partner ErbB receptor alters the consequences of stimulation with a given EGFR ligand by coupling to different signaling pathways and biological responses than EGFR homodimers.

Numerous studies indicate that different EGFR ligands induce distinct biological responses and patterns of EGFR coupling to signaling pathways. For example, TGF α and AREG are more effective stimuli of EGFR coupling to biological responses associated with tumor cell metastasis (motility, invasiveness, etc.) than is EGF. These biological differences appear to be due to differences in the sites of agonist-induced EGFR tyrosine phosphorylation. EGF stimulates greater phosphorylation of EGFR Tyr1045 than does AREG. Thus, EGF stimulates greater EGFR ubiquitination and turnover than does AREG, presumably because of increased EGFR coupling to the ubiquitin ligase c-Cbl. Moreover, the duration of EGFR coupling to MAPK and PLC γ signaling is greater following stimulation with AREG than with EGF [38–43].

The mechanism by which different ligands cause phosphorylation of distinct sets of EGFR tyrosine residues is unclear. However, the crystal structure of the EGFR extracellular domain dimer when bound with EGF is distinct from the crystal structure of the EGFR extracellular domain when bound with TGF α . Thus, ligand-specific differences in the juxtapositioning of the receptor monomers within the receptor dimer may lead to differences in receptor tyrosine residue availability to the receptor kinase domain for phosphorylation [17].

2. Manuscript body

2.1. EGFR and primary breast tumors

The roles that EGFR and its ligands play in breast cancer have been a subject of intensive study and controversy. Some retrospective immunohistochemical studies have indicated that EGFR overexpression in primary tumors is an indicator of poor prognosis [44–47], whereas other similar studies have failed to establish

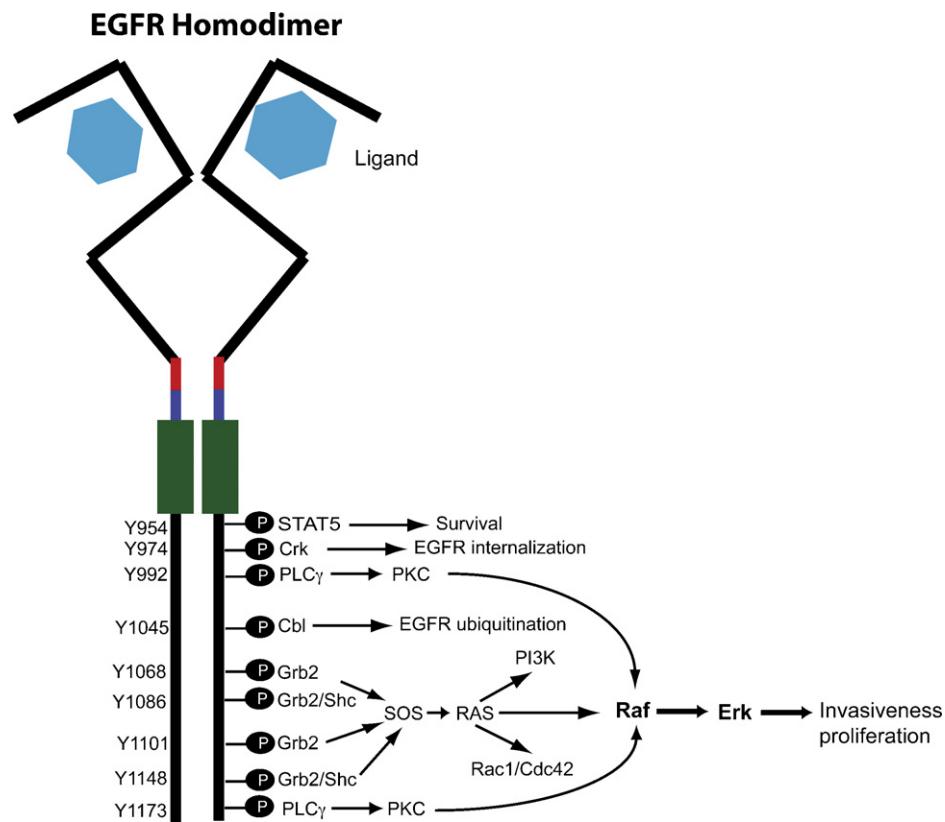


Fig. 1. The liganded EGFR homodimer possess multiple sites of tyrosine phosphorylation and couples to multiple signaling effectors. A schematic representation of the liganded EGFR homodimer is shown. The light blue hexagons represent the ligand. EGFR is depicted by a black line. Red and blue overlays represent the transmembrane and juxtamembrane domains, respectively. Green boxes represent the tyrosine kinase domains. Sites of cytosolic tyrosine (Y) phosphorylation are indicated, as are cytosolic effector proteins that bind to these phosphorylated tyrosine residues and some of the effector signaling pathways.

such a link [10,48]. Collectively, these studies suggest that EGFR is expressed in 18–35% of breast cancers but is not overexpressed relative to the normal breast epithelia [49]. Of course, because increased EGFR signaling is commonly associated with increased EGFR turnover, immunohistochemical analyses of EGFR protein expression may not be ideal for evaluating the role that EGFR may be playing in breast malignancies.

Initial studies have suggested that expression of EGF, TGF α or AREG is associated with larger and more aggressive tumors [9,50,51]. However, more extensive studies have failed to link ligand expression to prognosis [49,52]. This apparent dichotomy may be explained by the fact that immunohistochemical analyses of ligand expression in tumor samples primarily detects the immature, transmembrane form of the ligand, whereas signaling might be driven largely by the mature soluble form of the ligand.

2.1.1. Triple-negative, basal breast tumors

The development of platforms capable of simultaneously evaluating gene expression from a large portion of the genome has led to the identification of gene expression profiles that classify breast cancers. This has yielded further insights into the roles that EGFR and EGFR ligands may play in breast cancer. Basal-type breast cancers express markers frequently found in cells that are in contact with the basement membrane. Such markers include keratin 5 and 17 (basal keratins), P-cadherin, and troponin [53–56]. Basal-type breast cancers are associated with large size, high tumor grade, poor survival, and increased frequency of distant metastases [56]. These tumors typically lack expression of the estrogen receptor-alpha (ER α), progesterone receptor, and Erbb2. Thus, basal tumors are frequently referred to as “triple-negative” breast tumors [57].

Given the relative aggressiveness of these tumors and the absence of targeted therapeutics for treating these tumors, the identification of targets for treating these tumors is a priority.

Gene expression profiling and immunohistochemical studies have indicated that 50–70% of basal breast tumors exhibit EGFR expression [58]. Moreover, our preliminary analyses of breast cancer transcriptome datasets GSE2034 [59], GSE2603 [60], and GSE12276 [61] from the NCBI Gene Expression Omnibus reveal that the EGFR ligand TGF α and the EGFR/ErbB3/ErbB4 ligand NRG2 β are expressed at significantly higher levels in ER α -negative tumors than in ER α -positive tumors. Likewise, the expression of ADAMs and MMPs responsible for maturation (cleavage) of EGFR ligands is higher in ER α -negative tumors than in ER α -positive tumors. A low level of EGFR expression in basal tumors correlates with a reduced incidence of metastases [62]. Similarly, EGFR expression in basal tumors correlates with TGF α and ADAM-17 expression [63]. Thus, a sizable fraction of basal breast cancers appear to exhibit autocrine TGF α -EGFR signaling and this may account for the poor prognosis associated with these tumors [63].

In contrast, ER α -positive tumors tend to exhibit elevated AREG expression but no increase in EGFR expression [59–61]. This pattern of expression is similar to that exhibited by the normal mammary epithelia, in which ER α -positive cells exhibit little EGFR expression but do express AREG [64]. The AREG findings are consistent with previous reports from breast cancer cell lines that indicate this ligand is an estrogen regulated gene, but can be activated by several other pathways present in both ER α -positive and negative cancers [65,66]. One possible interpretation of the molecular profile data is that ER α -positive breast cancer would lack high levels of autocrine EGFR signaling, but could engage in paracrine EGFR sig-

naling with fibroblast-like cells in the microenvironment through AREG.

2.1.2. Resistance to antiestrogens

The estrogen receptor partial agonist tamoxifen (Tam) is commonly used to treat ER α -positive breast cancer in both pre- and post-menopausal women. However, a significant fraction of ER $^+$ tumors exhibit intrinsic resistance to Tam and in many patients responsiveness of ER α -positive tumors to Tam is of limited duration due to acquired resistance [67,68]. Indeed, many ER α $^+$ tumors acquire complete resistance to Tam, resulting in a restoration of tumor growth and metastasis [67,68].

Tam resistance may arise through overexpression or phosphorylation of the ER α co-activator AIB1/SRC-3 (amplified in breast cancer 1/steroid hormone receptor co-activator 3) [67,68]. This alters the effects of Tam on ER α -mediated gene expression, leading to Tam stimulation of mitogenic signaling pathways [67,68]. Signaling pathways downstream of several different tyrosine kinases induce phosphorylation of AIB1, suggesting that EGFR signaling may cause Tam resistance via this mechanism [67–69].

Tyrosine phosphorylation of ER α causes tamoxifen resistance by enabling estrogen-independent ER α -mediated gene expression [69]. A number of different tyrosine kinases may catalyze ER tyrosine phosphorylation, including ErbB2 [68,69]. Because ErbB2 is a common heterodimerization partner of EGFR, ligand-induced EGFR signaling may contribute to ER tyrosine phosphorylation and tamoxifen resistance.

Fulvestrant (Faslodex $^{\circledR}$; ICI 182,780) triggers rapid ER α degradation via the proteasome and is frequently used to treat receptor positive, tamoxifen-resistant tumors [68]. However, acquired resistance frequently arises, limiting the utility of this approach [68]. Chronic treatment of ER α -positive breast tumor cell lines with fulvestrant leads to clones that display resistance to fulvestrant. These models of acquired resistance typically display a loss of ER α -expression and elevated EGFR or ErbB2 expression and receptor tyrosine phosphorylation [70,71]. These cell lines also display elevated TGF α expression and retain AREG expression [70,71]. These data suggest that enhanced autocrine EGFR/ErbB2 signaling may compensate for the loss of ER expression and signaling in fulvestrant-resistant breast tumors. However, this hypothesis has yet to be tested in breast cancer patient samples.

2.1.3. Breast cancer stem cells

Solid tumors typically consist of a heterogeneous mix of cellular phenotypes that include poorly differentiated cells that undergo rapid cell division, differentiated cells that are incapable of cell division, and quiescent cells that possess the capacity for self-renewal and can give rise to the other types of tumor cells. This self-renewal and pluripotency have led this category of cells to be called cancer stem cells or stem-like cancer cells (Fig. 2) [72,73].

Breast cancer cells that have been isolated from pleural effusions exhibit a high level of CD44 expression and a low level of CD24 expression [74]. While these cells display a homogenous phenotype, they are extraordinarily efficient at forming phenotypically heterogeneous tumors in immunocompromised mice. Moreover, these cells readily form colonies in suspension cultures and exhibit very aggressive behaviors in metastasis and invasion assays [74]. Thus, these CD44 $^+$ /CD24 $^-$ breast tumor cells exhibit characteristics of tumor stem cells. ALDH1 has also emerged as a marker of tumor cells that exhibit stem-like characteristics [75,76].

There is no direct evidence indicating that EGFR and its ligands are involved in the establishment or maintenance of breast tumor stem cells. However, stem-like tumor cells are much more rare in ER α -positive breast tumors and breast cell lines (which typically have little EGFR and ErbB2 expression) than in triple-negative breast tumors and breast cell lines (which typically exhibit

elevated EGFR expression) [76]. Ligand-induced EGFR signaling is required for stem-like breast tumor cells (including those derived from DCIS tumors) to form colonies in semi-solid medium [77]. Overexpression of ErbB2 in mammary epithelial cells and breast cancer cell lines increases the fraction of cells that display stem-like properties [78]. Finally, a preliminary report from a small clinical trial indicates that the dual specificity EGFR/ErbB2 tyrosine kinase inhibitor lapatinib reduces the number of CD44 $^+$ /CD24 $^-$ cells found in breast tumor specimens [79]. These reports provide intriguing hints that the ligand-induced EGFR/ErbB2 signaling may play a substantial role in establishing and maintaining breast cancer stem-like cells. Nonetheless, additional direct experimentation is necessary to evaluate this hypothesis.

2.2. EGFR and bone metastasis

The most common metastasis site of breast cancer is the bone [80]. Nearly 70% of invasive breast cancer cases result in metastasis to the bone and generate severe pain and disability in the patient [80]. Destruction of bone matrix is responsible for the fractures and bone pain associated with advanced breast cancer [81]. The majority of tumors that metastasize to bone are ER α -positive [82], but there are a fraction of ER α -negative tumors that also metastasize to this location [83]. Bone metastases were largely refractory to the traditional systemic approaches (radiation therapy and chemotherapy) used to treat advanced breast cancer [80,81,84]. Recently, the integration of the fields of basic bone cell biology and cancer biology has produced insights that have generated new and partially effective therapeutic approaches to this devastating form of metastasis. Agents such as bisphosphonates reduce bone destruction and tumor growth by targeting the bone microenvironment rather than the tumor [84]. Recently, EGFR signaling has come into focus as a potential microenvironment target that could be exploited to reduce the morbidity associated with this form of metastasis.

Metastasis to any organ features invasion of cancer cells through normal tissue into the blood stream (initiation), extravasation and infiltration of a distant tissue (progression), and growth of a destructive colony within the new context (virulence) [85]. The genes that mediate these events are likely to be dispensable for primary tumor initiation and growth and may or may not be part of gene expression profiles exhibited by the primary tumor [85]. We have analyzed breast cancer transcriptome datasets from the NCBI Gene Expression Omnibus to compare the patterns of ErbB receptor and ligand in primary tumors that ultimately produced bone metastasis to the patterns found in tumors that failed to metastasize or produced metastases to other sites [59–61]. We have also compared ErbB receptor expression in a small set of bone metastasis samples with ErbB receptor expression in breast cancer samples removed from the lung, brain and liver [83]. ErbB2 expression was lower in those ER α -negative tumors that produced bone metastases than in tumors that did not metastasize to bone, which suggests that tumors that overexpress ErbB2 typically metastasize to visceral sites [86]. Surprisingly, AREG expression was significantly lower in ER α -negative tumors that ultimately metastasized to bone than in other ER α -negative tumors. However, we found little additional evidence for differential expression of ErbB family receptors. These findings suggest that EGFR signaling may be dysregulated in bone metastases through post-transcriptional events. As indicated below, several emerging lines of evidence involving ligand-activating proteases support a role for the EGFR signaling in bone metastasis.

2.2.1. Latent bone colonization by breast tumor cells

Frequently, bone metastasis arise in breast cancer patients years after the identification and treatment of the primary tumor [87]. This implies that breast cancer cells remain dormant or indolent

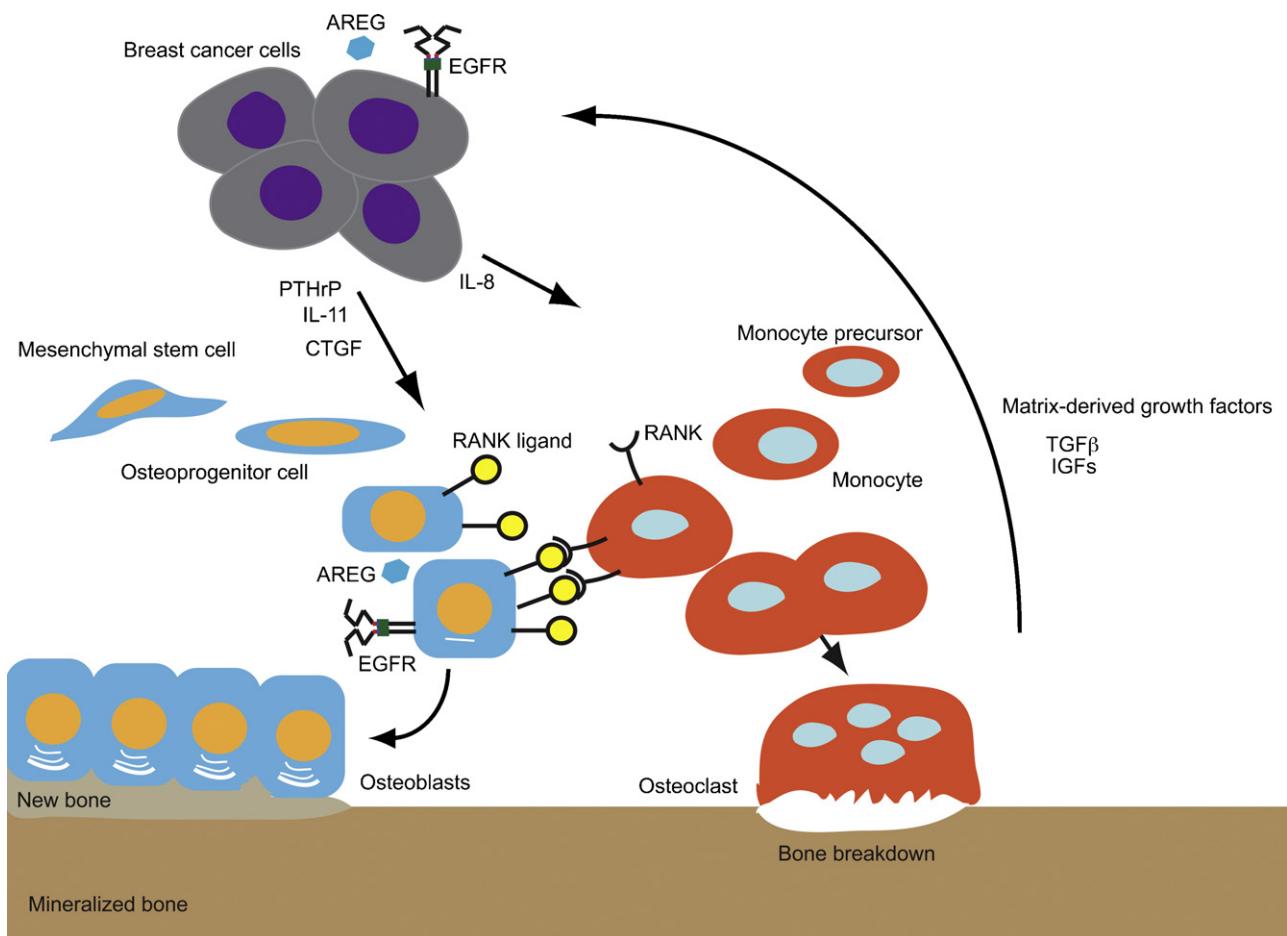


Fig. 2. Complex interactions of tumor and bone cells regulate bone biosynthesis and breakdown. Breast cancer cells express PTHrP, IL-11, and CTGF, which stimulate RANK ligand (RANKL) expression by cells of the osteoblast lineage. RANKL binding to RANK on monocytes stimulates their differentiation to active osteoclasts and consequent bone breakdown. Breast cancer cells also express IL-8, which directly stimulates monocyte production and leads to increased osteoclast formation. Breakdown of the bone matrix by osteoclasts releases TGF β and IGFs, which stimulate tumor cell survival, proliferation, and release of osteolytic factors. Both breast cancer cells and cells of the osteoblast lineage express EGFR and the EGFR ligand AREG.

within the body. Over the past two decades methodology has been developed to identify dormant/latent tumor cells within patients. Individual or small groups of tumor cells found in the bone marrow of patients who lack discernable bone metastases are termed disseminated tumor cells (DTCs) [87]. The presence of DTCs in the bone marrow is predictive of metastatic disease both in the bone and at other sites [87–89]. The vast majority of DTCs present in the bone marrow of breast cancer patients are CD44 $^{+}$ /CD24 $^{-}$, making them reminiscent of stem-like breast cancer cells [90]. However, elevated EGFR and ErbB2 are also markers for DTCs [91,92]. This suggests that ErbB receptors play a role in the establishment or maintenance of stem-like breast cancer cells, but there is no further information regarding potential function of ErbB receptors in the infiltration of breast cancer cells into bone, or regarding their possible impact on latency/indolence [93].

2.2.2. Bone metastasis: a vicious cycle

Much of the advances in the understanding of breast cancer colonization of bone has stemmed from studies of the MDA-MB-231 ER α -negative breast cancer cell line in bone xenografts. MDA-MB-231 cells possess a basal phenotype [94] and various bone-seeking sublines have been developed to dissect the molecular and cellular regulators of osteolytic growth of this cell line [95,96]. On the basis of these studies, the concept of “the vicious cycle” of tumor cell growth linked to bone destruction has been developed [96]. This model holds that breast cancer cells direct the resident

cells of bone to uncouple the physiological linkage between bone matrix destruction and new bone formation [96]. The MDA-MB-231 cells produce cytokines and growth factors that engage in paracrine signaling with osteoclasts, cells that dissolve bone matrix, and osteoblasts, which are responsible for bone formation [96,97]. Osteoclast formation is mediated mainly through RANK (receptor activator of nuclear factor β -ligand) and its agonist RANKL (RANK ligand), the latter of which is produced by osteoblasts and bone marrow stromal cells [93,96]. Osteoblastic cells also produce a soluble RANKL sink called osteoprotegerin (OPG) [80,93]. Thus, osteoclast formation is influenced by the balance between RANKL and OPG in the bone microenvironment [96]. In addition, osteoblasts produce colony-stimulating factor (CSF-1), which recruits monocytes from bone marrow progenitors that ultimately can be differentiated into osteoclasts in the presence of high levels of RANKL [96,98,99]. In the MDA-MB-231 xenograft models, the breast cancer cells produce several growth factors and cytokines that perturb the RANKL/OPG ratio and increase the number of monocytes that can be differentiated to osteoclasts [95,97,99]. The osteoclast-mediated destruction of bone releases growth factors embedded in the bone matrix. These stimulate their cognate receptors on the cancer cells, resulting in increased tumor cell proliferation and production of cytokines that skew the RANKL/OPG ratio toward increased osteoclastogenesis, thereby propagating a vicious cycle of tumor cell proliferation and bone destruction [97,100]. It should be noted that this model is based on the activi-

ties of the ER α -negative MDA-MB-231 breast tumor cell line, and it is unclear whether all of the specific molecules and cellular interactions apply to the more common form of disease progression that arise from ER α -positive breast tumors.

2.2.3. EGFR and osteolysis

There is growing evidence suggesting that EGFR signaling in osteoblasts directly contributes to osteolysis or bone resorption. EGFR is expressed by cultured osteoblasts, but not osteoclasts or monocytes [101,102]. Furthermore, EGF, TGF α , and MDA-MB-231 cells (which express various ErbB ligands) stimulate bone turnover and osteoclastogenesis in various model systems [103–106]. This osteoclastogenesis is accompanied by decreased OPG expression and minimal change in RANKL expression by the bone cells [106]. EGFR TKIs inhibit CSF-1 and RANKL production from human bone marrow stromal cells and osteoclast formation *in vitro* [107]. These studies clearly support the concept that EGFR signaling within the osteoblast promotes osteoclastogenesis through perturbation of the RANKL/OPG balance.

2.2.4. EGFR and osteoblast function

Studies of bone biology suggest additional roles for EGFR ligands in the pathogenesis of osteolytic lesions. Parathyroid hormone (PTH), the main serum calcium regulator, stimulates AREG gene transcription 10–20-fold and stimulates more modest increases in transcription of the TGF α and HB-EGF genes [108,109]. The PTH receptor, like other serpentine G-protein-coupled receptors (GPCRs), appears to be coupled to proteases (such as ADAM-17) that cleave ErbB receptor ligand precursors and enable the release of the mature, soluble ligands [110].

Exogenous EGFR ligands stimulate the proliferation of osteoblasts, inhibit their differentiation, and decrease their mineralization [109]. Moreover, 4-week-old transgenic mice lacking AREG expression exhibit less trabecular bone in the tibia than do wild-type littermates [109]. Thus, EGFR signaling may mediate the impact of PTH on the recruitment and expansion of cells committed to the osteoblast lineage, whereas excessive ligand signaling could prevent these cells from undergoing terminal differentiation and forming mineralized bone [109]. The uncoupling of bone formation from the accelerated bone resorption would be a key feature of disease states like breast cancer-induced osteolysis (Fig. 3).

2.2.5. EGFR and PTHrP

In the MDA-MB-231 model, PTH receptor signaling is one of the key events in regulating the vicious cycle of breast cancer osteolysis and colonization [111]. MDA-MB-231 cells express parathyroid hormone-related peptide (PTHrP), another PTH receptor agonist that stimulates RANKL expression and inhibits OPG expression in cells of the osteoblast lineage [111]. The pattern of PTHrP expression by breast cancers at various stages of progression resembles that displayed by metastasis virulence factors [85]. PTHrP expression is lower in primary breast cancers that ultimately metastasize to bone than in other primary breast tumors; however, PTHrP expression is very high among metastatic tumor cells within the bone microenvironment [112–115]. PTHrP gene expression in these metastatic tumor cells appears to be stimulated by TGF β released from the bone matrix via osteoclast activity [96,100]. Nonetheless, the signaling between the PTHrP and the EGFR system is not simply directed from cancer cell to the microenvironment. In many epithelial cells EGFR is coupled to PTHrP gene expression [116–118]. In fact, an autocrine loop of AREG-EGFR signaling activates PTHrP transcription in the MDA-MB-231 line *in vitro* [119]. Thus, autocrine EGFR stimulation in breast cancer cells may contribute to the release of cytokines, such as PTHrP, that directly

perturb the RANKL/OPG balance and indirectly stimulate EGFR signaling within cells of the osteoblast lineage.

2.2.6. EGFR ligands and activating proteases as bone metastasis virulence factors

Analysis of MDA-MB-231 subclones identified 11 genes whose overexpression is specific to clones that readily colonize the bone and form aggressive osteolytic lesions [95]. Moreover, combinations of 3 of these genes are sufficient to induce osteolytic growth by parental MDA-MB-231 cells. Thus, these 11 genes appear to influence distinct events in the process of bone metastasis. These 11 genes include IL-11, which alters the RANKL/OPG balance, and connective tissue factor, which stimulates osteoblast proliferation. These 11 genes also include the proteases MMP1 and ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin motifs), whose roles in bone metastasis were not readily apparent [95].

Overexpression of MMP1 and ADAMTS-1 in MDA-MB-231 cells dramatically increased AREG shedding and resulted in a cell line that formed more aggressive osteolytic lesions in the bone. Conditioned medium from the MDA-MB-231/ADAMTS-1/MMP1 cells altered the RANKL/OPG balance in a primary mouse bone cell culture and enhanced osteoclastogenesis. This enhanced osteoclastogenesis could be inhibited by the EGFR TKI gefitinib or by the anti-EGFR antibody cetuximab. Moreover, these agents (gefitinib 100 mg/kg daily or cetuximab 100 mg/kg weekly) prevented MDA-MB-231/ADAMTS-1/MMP1 cells from stimulating the formation of osteolytic lesions in the bone of immunocompromised mice injected with these cells [120]. These findings suggest that EGFR ligands or the proteases that regulate their availability can serve as breast cancer metastasis virulence factors and that metastasis could be blocked by EGFR antagonists that have no apparent direct effect on the breast tumor cells themselves.

This finding that AREG expression is necessary but not sufficient for MDA-MB-231 cells to colonize the bone is consistent with the observation that AREG expression is lower in ER α -negative breast tumors that ultimately metastasized to bone than in ER α -negative breast tumor that failed to metastasize to bone. Presumably, differences in the ability of breast tumor cells to colonize bone is regulated by proteases cleave AREG and enable it to stimulate EGFR signaling. Indeed, elevated expression of ADAMTS-1 and MMP1 is observed in primary breast cancer tumors that ultimately metastasize to bone [63]. Furthermore, given that various GPCRs are coupled to increased activity of MMPs and ADAMs, we speculate that increased signaling by GPCRs on tumor cells in the bone microenvironment may contribute to bone colonization by coupling to increased activity MMPs and ADAMs [121,122].

To summarize, the complex post-transcriptional regulation of EGFR ligand processing and receptor interactions provides mechanisms through which EGFR coupling to bone colonization may be enhanced. Thus, numerous gene products that contribute to EGFR signaling in breast tumor cells or osteoblasts may function as bone metastasis virulence factors. (1) The combination of an EGFR ligand (such as AREG) and an active shedding protease (such as MMP1 or ADAMTS-1) in breast tumor cells could activate paracrine EGFR signaling in osteoblasts, resulting in reduced OPG expression, increased osteoclastogenesis and decreased bone mineralization. (2) Autocrine EGFR signaling in the tumor cell could couple to PTHrP expression and release by tumor cells, leading to increased RANKL and decreased OPG expression in osteoblasts. (3) PTHrP released by tumor cells could also stimulate AREG expression and ADAM17 activity in osteoblasts, leading to increased EGFR signaling in the osteoblasts. Thus, PTHrP could play a central role in two pathways that independently lead to a robust alteration of the RANKL/OPG balance to favor osteoclast formation and osteolytic activity.

The multiple mechanisms by which MDA-MB-231 cells can stimulate EGFR coupling to osteolytic effects in the bone microen-

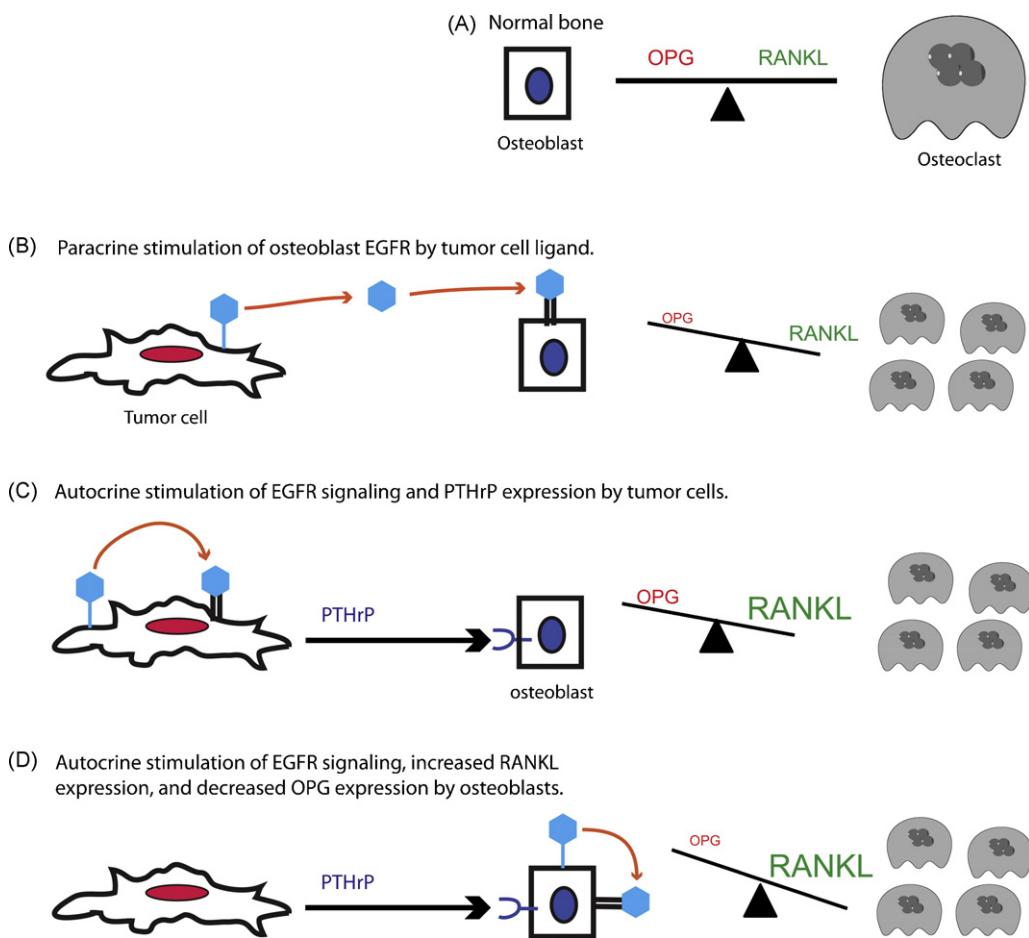


Fig. 3. EGFR may play multiple roles in breast cancer-induced osteolysis. (A) In normal bone RANKL stimulation of osteoclast-mediated bone turnover and is balanced by the OPG antagonist of RANKL. (B) An EGFR ligand (light blue hexagon) expressed and shed by tumor cells may stimulate paracrine signaling by EGFR (double black bars) expressed by osteoblasts. This would inhibit OPG expression by osteoblasts, leading to increased RANKL stimulation of RANK expressed by osteoclasts and increased osteoclast-mediated bone turnover. (C) An EGFR ligand expressed and shed by tumor cells may stimulate autocrine signaling by EGFR expressed by the tumor cells, leading to PTHrP expression by these tumor cells. This stimulates RANKL expression and inhibits OPG expression by osteoblasts, again leading to increased RANKL stimulation of RANK expressed by osteoclasts and increased osteoclast-mediated bone turnover. (D) PTHrP expressed by tumor cells can also stimulate expression of an EGFR ligand by osteoblasts, leading to autocrine EGFR signaling and coupling to increased RANKL expression and decreased OPG expression in osteoblasts. Again, this leads to increased RANKL stimulation of RANK expressed by osteoclasts and increased osteoclast-mediated bone turnover.

vironment indicate that this pathway may be a major component of the pathogenesis of osteolytic lesions triggered by this ER α -negative breast cancer line. Moreover, AREG transcription is positively regulated by ER α in the mouse mammary gland and breast cancer cells [64,66]. Thus, deregulated signaling through the AREG-EGFR pathway may be a general mechanism by which multiple types of breast cancer form osteolytic bone metastases.

Small-molecule EGFR tyrosine kinase inhibitors and antagonistic anti-EGFR antibodies have exhibited little effect on primary tumor growth or patient outcome in breast cancer monotherapy clinical trials. One possibility is that anti-EGFR agents will be effective against bone metastases, but will have little effect on the primary tumor [97,120,123–125]. The other possibility is that these agents may be effective only as part of combination therapy regimens. Indeed, emerging data appear to support this possibility, particularly in advanced ER α -positive breast cancers [126–128].

Acknowledgements

The authors acknowledge support of NIH grants to DJR (R01CA114209) and the Purdue University Center for Cancer Research (P30CA023168). JF has been supported by grants from

Susan G. Komen for the Cure (KG081561) and the Indiana University Breast Cancer Research Program (29-875-62). JLG has been supported by Walther Cancer Center and Susan G. Komen for the Cure postdoctoral fellowships. NKN has been supported by a Department of Defense Breast Cancer Research Program predoctoral fellowship. Support from the NCI (U54CA113001) to KPN is gratefully acknowledged.

References

- [1] Carney W, Leitzel K, Ali S, Neumann R, Lipton A. HER-2 therapy. HER-2/neu diagnostics in breast cancer. *Breast Cancer Res* 2007;9:207.
- [2] Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 2005;5:341–54.
- [3] Jones FE. HER4 intracellular domain (4ICD) activity in the developing mammary gland and breast cancer. *J Mammary Gland Biol Neoplasia* 2008;13:247–58.
- [4] O'Donovan N, Crown J. EGFR and HER-2 antagonists in breast cancer. *Anti-cancer Res* 2007;27:1285–94.
- [5] Roskoski Jr R. The ErbB/HER receptor protein-tyrosine kinases and cancer. *Biochem Biophys Res Commun* 2004;319:1–11.
- [6] Spector N, Xia W, El-Harrar I, Yarden Y, Bacus S. HER2 therapy. Small molecule HER-2 tyrosine kinase inhibitors. *Breast Cancer Res* 2007;9:205.
- [7] Stern D. ERBB3/HER3 and ERBB2/HER2 duet in mammary development and breast cancer. *J Mammary Gland Biol Neoplasia* 2008;13:215–23.

- [8] Sundvall M, Iljin K, Kilpinen S, Sara H, Kallioniemi OP, Elenius K. Role of ErbB4 in breast cancer. *J Mammary Gland Biol Neoplasia* 2008;13:259–68.
- [9] Suo Z, Risberg B, Karlsson MG, Villman K, Skovlund E, Nesland JM. The expression of EGFR family ligands in breast carcinomas. *Int J Surg Pathol* 2002;10:91–9.
- [10] Tsutsui S, Ohno S, Murakami S, Hachitanda Y, Oda S. Prognostic value of epidermal growth factor receptor (EGFR) and its relationship to the estrogen receptor status in 1029 patients with breast cancer. *Breast Cancer Res Treat* 2002;71:67–75.
- [11] Valabrega G, Montemurro F, Aglietta M. Trastuzumab: mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer. *Ann Oncol* 2007;18:977–84.
- [12] Wieduwilt MJ, Moasser MM. The epidermal growth factor receptor family: biology driving targeted therapeutics. *Cell Mol Life Sci* 2008;65: 1566–84.
- [13] Zaczek A, Brandt B, Bielawski KP. The diverse signaling network of EGFR, HER2, HER3 and HER4 tyrosine kinase receptors and the consequences for therapeutic approaches. *Histol Histopathol* 2005;20:1005–15.
- [14] Campbell MR, Amin D, Moasser MM. HER3 comes of age: new insights into its functions and role in signaling, tumor biology, and cancer therapy. *Clin Cancer Res* 2010;16:1373–83.
- [15] Lee-Hoeffler ST, Crocker L, Yao E, Pham T, Munroe X, Hoeflich KP, et al. A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy. *Cancer Res* 2008;68:5878–87.
- [16] Riese 2nd DJ, Stern DF. Specificity within the EGF family/ErbB receptor family signaling network. *Bioessays* 1998;20:41–8.
- [17] Wilson KJ, Gilmore JL, Foley J, Lemmon MA, Riese 2nd DJ. Functional selectivity of EGF family peptide growth factors: implications for cancer. *Pharmacol Ther* 2009;122:1–8.
- [18] Mill CP, Chester JA, Riese DJ. EGFR may couple moderate alcohol consumption to increased breast cancer risk. *Breast Cancer (London)* 2009;2009:31–8.
- [19] Ohtsu H, Dempsey PJ, Eguchi S. ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors. *Am J Physiol Cell Physiol* 2006;291:C1–10.
- [20] Shah BH, Catt KJ. TACE-dependent EGF receptor activation in angiotensin-II-induced kidney disease. *Trends Pharmacol Sci* 2006;27:235–7.
- [21] Bhola NE, Grandis JR. Crosstalk between G-protein-coupled receptors and epidermal growth factor receptor in cancer. *Front Biosci* 2008;13:1857–65.
- [22] Daub H, Weiss FU, Wallasch C, Ullrich A. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* 1996;379:557–60.
- [23] Tada H, Sasada R, Kawaguchi Y, Kojima I, Gullick WJ, Salomon DS, et al. Processing and juxtaactive activity of membrane-anchored betacellulin. *J Cell Biochem* 1999;72:423–34.
- [24] Dobashi Y, Stern DF. Membrane-anchored forms of EGF stimulate focus formation and intercellular communication. *Oncogene* 1991;6:1151–9.
- [25] Dong J, Opresko LK, Chrisler W, Orr G, Quesenberry RD, Lauffenburger DA, et al. The membrane-anchoring domain of epidermal growth factor receptor ligands dictates their ability to operate in juxtaactive mode. *Mol Biol Cell* 2005;16:2984–98.
- [26] Riese 2nd DJ, Gallo RM, Settleman J. Mutational activation of ErbB family receptor tyrosine kinases: insights into mechanisms of signal transduction and tumorigenesis. *Bioessays* 2007;29:558–65.
- [27] Lemmon MA. Ligand-induced ErbB receptor dimerization. *Exp Cell Res* 2009;315:638–48.
- [28] Schulze WX, Deng L, Mann M. Phosphotyrosine interactome of the ErbB-receptor kinase family. *Mol Syst Biol* 2005;1:0008.
- [29] Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* 2010;141:1117–34.
- [30] Sorkin A, Mazzotti M, Sorkina T, Scotto L, Beguinot L. Epidermal growth factor receptor interaction with clathrin adaptors is mediated by the Tyr974-containing internalization motif. *J Biol Chem* 1996;271:13377–84.
- [31] Keilhack H, Tenev T, Nyakatura E, Godovac-Zimmermann J, Nielsen L, Seedorf K, et al. Phosphotyrosine 1173 mediates binding of the protein-tyrosine phosphatase SHP-1 to the epidermal growth factor receptor and attenuation of receptor signaling. *J Biol Chem* 1998;273:24839–46.
- [32] Tenev T, Keilhack H, Tomic S, Stoyanov B, Stein-Gerlach M, Lammers R, et al. Both SH2 domains are involved in interaction of SHP-1 with the epidermal growth factor receptor but cannot confer receptor-directed activity to SHP-1/SHP-2 chimera. *J Biol Chem* 1997;272:5966–73.
- [33] Earp HS, Dawson TL, Li X, Yu H. Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research. *Breast Cancer Res Treat* 1995;35:115–32.
- [34] Wada T, Qian XL, Greene MI. Intermolecular association of the p185neu protein and EGF receptor modulates EGF receptor function. *Cell* 1990;61:1339–47.
- [35] Gulliford TJ, Huang GC, Ouyang X, Epstein RJ. Reduced ability of transforming growth factor-alpha to induce EGF receptor heterodimerization and down-regulation suggests a mechanism of oncogenic synergy with ErbB2. *Oncogene* 1997;15:2219–23.
- [36] Wang Z, Zhang L, Yeung TK, Chen X. Endocytosis deficiency of epidermal growth factor (EGF) receptor-ErbB2 heterodimers in response to EGF stimulation. *Mol Biol Cell* 1999;10:1621–36.
- [37] Hendriks BS, Wiley HS, Lauffenburger D. HER2-mediated effects on EGFR endosomal sorting: analysis of biophysical mechanisms. *Biophys J* 2003;85:2732–45.
- [38] Gilmore JL, Scott JA, Bouizar Z, Robling A, Pitfield SE, Riese 2nd DJ, et al. Amphiregulin-EGFR signaling regulates PTHrP gene expression in breast cancer cells. *Breast Cancer Res Treat* 2008;110:493–505.
- [39] Levkowitz G, Waterman H, Zamir E, Kam Z, Oved S, Langdon WY, et al. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev* 1998;12:3663–74.
- [40] Stern KA, Place TL, Lill NL. EGF and amphiregulin differentially regulate Cbl recruitment to endosomes and EGF receptor fate. *Biochem J* 2008;410:585–94.
- [41] Willmarth NE, Baillo A, Dziubinski ML, Wilson K, Riese 2nd DJ, Ethier SP. Altered EGFR localization and degradation in human breast cancer cells with an amphiregulin/EGFR autocrine loop. *Cell Signal* 2009;21:212–9.
- [42] Gilmore JL, Gonterman RM, Menon K, Lorch G, Riese 2nd DJ, Robling A. Reconstitution of amphiregulin-epidermal growth factor receptor signaling in lung squamous cell carcinomas activates PTHrP gene expression and contributes to cancer-mediated diseases of the bone. *Mol Cancer Res* 2009;7: 1714–28.
- [43] Joslin EJ, Opresko LK, Wells A, Wiley HS, Lauffenburger DA. EGF-receptor-mediated mammary epithelial cell migration is driven by sustained ERK signaling from autocrine stimulation. *J Cell Sci* 2007;120:3688–99.
- [44] Garcia Castro C, Ravina M, Castro V, Salido EC. Expression of epidermal growth factor receptor (proto-oncogene c-erbB-1) and estrogen receptor in human breast carcinoma. An immunocytochemical study of 70 cases. *Arch Gynecol Obstet* 1993;252:169–77.
- [45] Martinazzi M, Crivelli F, Zampatti C, Martinazzi S. Epidermal growth factor receptor immunohistochemistry in different histological types of infiltrating breast carcinoma. *J Clin Pathol* 1993;46:1009–10.
- [46] Newby JC, A'Hern RP, Leek RD, Smith IE, Harris AL, Dowsett M. Immunohistochemical assay for epidermal growth factor receptor on paraffin-embedded sections: validation against ligand-binding assay and clinical relevance in breast cancer. *Br J Cancer* 1995;71:1237–42.
- [47] Schroeder W, Biesterfeld S, Zillessen S, Rath W. Epidermal growth factor receptor-immunohistochemical detection and clinical significance for treatment of primary breast cancer. *Anticancer Res* 1997;17: 2799–802.
- [48] Ferrero JM, Ramaioli A, Largillier R, Formento JL, Francoual M, Ettore F, et al. Epidermal growth factor receptor expression in 780 breast cancer patients: a reappraisal of the prognostic value based on an eight-year median follow-up. *Ann Oncol* 2001;12:841–6.
- [49] Pawlowski V, Revillion F, Hebbar M, Hornez L, Peyrat JP. Prognostic value of the type I growth factor receptors in a large series of human primary breast cancers quantified with a real-time reverse transcription-polymerase chain reaction assay. *Clin Cancer Res* 2000;6:4217–25.
- [50] Ma L, de Roquancourt A, Bertheau P, Chevret S, Millot G, Sastre-Garau X, et al. Expression of amphiregulin and epidermal growth factor receptor in human breast cancer: analysis of autocrine and stromal–epithelial interactions. *J Pathol* 2001;194:413–9.
- [51] Soares R, Pereira MB, Silva C, Amendoeira I, Wagner R, Ferro J, et al. Expression of TGF-alpha and EGFR in breast cancer and its relation to angiogenesis. *Breast J* 2000;6:171–7.
- [52] Desruisseaux S, Palmari J, Giusti C, Romain S, Martin PM, Berthois Y. Clinical relevance of amphiregulin and VEGF in primary breast cancers. *Int J Cancer* 2004;111:733–40.
- [53] van't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002;415:530–6.
- [54] van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002;347:1999–2009.
- [55] Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001;98:10869–74.
- [56] Da Silva L, Clarke C, Lakhani SR. Demystifying basal-like breast carcinomas. *J Clin Pathol* 2007;60:1328–32.
- [57] Korschning E, Jeffrey SS, Meinerz W, Decker T, Boecker W, Buerger H. Basal carcinoma of the breast revisited: an old entity with new interpretations. *J Clin Pathol* 2008;61:553–60.
- [58] Burness ML, Grushko TA, Olopade OI. Epidermal growth factor receptor in triple-negative and basal-like breast cancer: promising clinical target or only a marker? *Cancer J* 2010;16:23–32.
- [59] Wang Y, Klijn JG, Zhang Y, Sieuwerts AM, Look MP, Yang F, et al. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* 2005;365:671–9.
- [60] Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, et al. Genes that mediate breast cancer metastasis to lung. *Nature* 2005;436:518–24.
- [61] Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, Nguyen DX, et al. Genes that mediate breast cancer metastasis to the brain. *Nature* 2009;459:1005–9.
- [62] Viale G, Rotmensz N, Maisonneuve P, Bottiglieri L, Montagna E, Luini A, et al. Invasive ductal carcinoma of the breast with the “triple-negative” phenotype: prognostic implications of EGFR immunoreactivity. *Breast Cancer Res Treat* 2009;116:317–28.
- [63] Kenny PA, Bissell MJ. Targeting TACE-dependent EGFR ligand shedding in breast cancer. *J Clin Invest* 2007;117:337–45.
- [64] Ciaroni L, Mallepell S, Brisken C. Amphiregulin is an essential mediator of estrogen receptor alpha function in mammary gland development. *Proc Natl Acad Sci USA* 2007;104:5455–60.

- [65] Rho JY, Wada-Kiyama Y, Onishi Y, Kiyama R, Sakuma Y. Expressional regulation of neuronal and cancer-related genes by estrogen in adult female rats. *Endocr Res* 2004;30:257–67.
- [66] Britton DJ, Hutcheson IR, Knowlden JM, Barrow D, Giles M, McClelland RA, et al. Bidirectional cross talk between ERalpha and EGFR signalling pathways regulates tamoxifen-resistant growth. *Breast Cancer Res Treat* 2006;96:131–46.
- [67] Fan P, Yue W, Wang JP, Aiyar S, Li Y, Kim TH, et al. Mechanisms of resistance to structurally diverse antiestrogens differ under premenopausal and postmenopausal conditions: evidence from *in vitro* breast cancer cell models. *Endocrinology* 2009;150:2036–45.
- [68] Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and anti-hormonal resistance in breast cancer. *J Clin Oncol* 2007;25:5815–24.
- [69] Santen RJ, Fan P, Zhang Z, Bao Y, Song RX, Yue W. Estrogen signals via an extra-nuclear pathway involving IGF-1R and EGFR in tamoxifen-sensitive and -resistant breast cancer cells. *Steroids* 2009;74:586–94.
- [70] Fan M, Yan PS, Hartman-Frey C, Chen L, Paik H, Oyer SL, et al. Diverse gene expression and DNA methylation profiles correlate with differential adaptation of breast cancer cells to the antiestrogens tamoxifen and fulvestrant. *Cancer Res* 2006;66:11954–66.
- [71] Frogne T, Benjaminsen RV, Sonne-Hansen K, Sorensen BS, Nexo E, Laenkholm AV, et al. Activation of ErbB3, EGFR and Erk is essential for growth of human breast cancer cell lines with acquired resistance to fulvestrant. *Breast Cancer Res Treat* 2009;114:263–75.
- [72] Dick JE. Stem cell concepts renew cancer research. *Blood* 2008;112:4793–807.
- [73] Dick JE. Looking ahead in cancer stem cell research. *Nat Biotechnol* 2009;27:44–6.
- [74] Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003;100:3983–8.
- [75] Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 2007;1:555–67.
- [76] Charafe-Jauffret E, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P, et al. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. *Cancer Res* 2009;69:1302–13.
- [77] Farnie G, Clarke RB, Spence K, Pinnock N, Brennan K, Anderson NG, et al. Novel cell culture technique for primary ductal carcinoma *in situ*: role of Notch and epidermal growth factor receptor signaling pathways. *J Natl Cancer Inst* 2007;99:616–27.
- [78] Korkaya H, Paulson A, Iovino F, Wicha MS. HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. *Oncogene* 2008;27:6120–30.
- [79] Schmidt C. Lapatinib study supports cancer stem cell hypothesis, encourages industry research. *J Natl Cancer Inst* 2008;100:694–5.
- [80] Guise TA, Mundy GR. Cancer and bone. *Endocr Rev* 1998;19:18–54.
- [81] Ali SM, Harvey HA, Lipton A. Metastatic breast cancer: overview of treatment. *Clin Orthop Relat Res* 2003;415:S132–7.
- [82] Clark GM, Sledge Jr GW, Osborne CK, McGuire WL. Survival from first recurrence: relative importance of prognostic factors in 1,015 breast cancer patients. *J Clin Oncol* 1987;5:55–61.
- [83] Zhang XH, Wang Q, Gerald W, Hudis CA, Norton L, Smid M, et al. Latent bone metastasis in breast cancer tied to Src-dependent survival signals. *Cancer Cell* 2009;16:67–78.
- [84] Diel IJ, Solomayer EF, Costa SD, Gollan C, Goerner R, Wallwiener D, et al. Reduction in new metastases in breast cancer with adjuvant clodronate treatment. *N Engl J Med* 1998;339:357–63.
- [85] Nguyen DX, Bos PD, Massaguer J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer* 2009;9:274–84.
- [86] Emi Y, Kitamura K, Shikada Y, Kakeji Y, Takahashi I, Tsutsui S. Metastatic breast cancer with HER2/neu-positive cells tends to have a morbid prognosis. *Surgery* 2002;131:S217–21.
- [87] Riethdorf S, Wikman H, Pantel K. Review: biological relevance of disseminated tumor cells in cancer patients. *Int J Cancer* 2008;123:1991–2006.
- [88] Fehm T, Mueller V, Marches R, Klein G, Gueckel B, Neubauer H, et al. Tumor cell dormancy: implications for the biology and treatment of breast cancer. *Apmis* 2008;116:742–53.
- [89] Wikman H, Vessella R, Pantel K. Cancer micrometastasis and tumour dormancy. *Apmis* 2008;116:754–70.
- [90] Balic M, Lin H, Young L, Hawes D, Giuliano A, McNamara G, et al. Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. *Clin Cancer Res* 2006;12:5615–21.
- [91] Braun S, Schlimok G, Heumos I, Schaller G, Riethdorf L, Riethmuller G, et al. Erbb2 overexpression on occult metastatic cells in bone marrow predicts poor clinical outcome of stage I–III breast cancer patients. *Cancer Res* 2001;61:1890–5.
- [92] Pantel K, Schlimok G, Braun S, Kutter D, Lindemann F, Schaller G, et al. Differential expression of proliferation-associated molecules in individual micrometastatic carcinoma cells. *J Natl Cancer Inst* 1993;85:1419–24.
- [93] Thomas RJ, Guise TA, Yin JJ, Elliott J, Horwood NJ, Martin TJ, et al. Breast cancer cells interact with osteoblasts to support osteoclast formation. *Endocrinology* 1999;140:4451–8.
- [94] Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* 2006;10:515–27.
- [95] Kang Y, Siegel PM, Shu W, Drobniak M, Kakonen SM, Cordon-Cardo C, et al. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 2003;3:537–49.
- [96] Guise TA, Mohammad KS, Clines G, Stebbins EG, Wong DH, Higgins LS, et al. Basic mechanisms responsible for osteolytic and osteoblastic bone metastases. *Clin Cancer Res* 2006;12:6213S–6S.
- [97] Guise TA. Breaking down bone: new insight into site-specific mechanisms of breast cancer osteolysis mediated by metalloproteinases. *Genes Dev* 2009;23:2117–23.
- [98] Li Z, Schem C, Shi Y, Medina D, Zhang M. Increased COX2 expression enhances tumor-induced osteoclastic lesions in breast cancer bone metastasis. *Clin Exp Metastasis* 2007;25:389–400.
- [99] Bredt MS, Gaddy-Kurten D, Mon-Foote T, Akel NS, Skinner RA, Nicholas RW, et al. Expression of interleukin 8 and not parathyroid hormone-related protein by human breast cancer cells correlates with bone metastasis *in vivo*. *Cancer Res* 2002;62:5571–9.
- [100] Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, et al. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* 1999;103:197–206.
- [101] Davideau JL, Sahlberg C, Thesleff I, Berdal A. EGF receptor expression in mineralized tissues: an *in situ* hybridization and immunocytochemical investigation in rat and human mandibles. *Connect Tissue Res* 1995;32:47–53.
- [102] Ng KW, Partridge NC, Niall M, Martin TJ. Epidermal growth factor receptors in clonal lines of a rat osteogenic sarcoma and in osteoblast-rich rat bone cells. *Calcif Tissue Int* 1983;35:298–303.
- [103] Guise TA, Yoneda T, Yates AJ, Mundy GR. The combined effect of tumor-produced parathyroid hormone-related protein and transforming growth factor-alpha enhance hypercalcemia *in vivo* and bone resorption *in vitro*. *J Clin Endocrinol Metab* 1993;77:40–5.
- [104] Ibbotson KJ, D'Souza SM, Ng KW, Osborne CK, Niall M, Martin TJ, et al. Tumor-derived growth factor increases bone resorption in a tumor associated with humoral hypercalcemia of malignancy. *Science* 1983;221:1292–4.
- [105] Ibbotson KJ, D'Souza SM, Smith DD, Carpenter G, Mundy GR. EGF receptor antiserum inhibits bone resorbing activity produced by a rat Leydig cell tumor associated with the humoral hypercalcemia of malignancy. *Endocrinology* 1985;116:469–71.
- [106] Zhu J, Jia X, Xiao G, Kang Y, Partridge NC, Qin L. EGF-like ligands stimulate osteoclastogenesis by regulating expression of osteoclast regulatory factors by osteoblasts: implications for osteolytic bone metastases. *J Biol Chem* 2007;282:26656–64.
- [107] Normanno N, De Luca A, Aldinucci D, Maiello MR, Mancino M, D'Antonio A, et al. Gefitinib inhibits the ability of human bone marrow stromal cells to induce osteoclast differentiation: implications for the pathogenesis and treatment of bone metastasis. *Endocr Relat Cancer* 2005;12:471–82.
- [108] Qin L, Qiu P, Wang L, Li X, Swarthout JT, Soteropoulos P, et al. Gene expression profiles and transcription factors involved in parathyroid hormone signaling in osteoblasts revealed by microarray and bioinformatics. *J Biol Chem* 2003;278:19723–31.
- [109] Qin L, Partridge NC. Stimulation of amphiregulin expression in osteoblastic cells by parathyroid hormone requires the protein kinase A and cAMP response element-binding protein signaling pathway. *J Cell Biochem* 2005;96:632–40.
- [110] Cole JA. Parathyroid hormone activates mitogen-activated protein kinase in opossum kidney cells. *Endocrinology* 1999;140:5771–9.
- [111] Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, et al. Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J Clin Invest* 1996;98:1544–9.
- [112] Southby J, Kissin MW, Danks JA, Hayman JA, Moseley JM, Henderson MA, et al. Immunohistochemical localization of parathyroid hormone-related protein in human breast cancer. *Cancer Res* 1990;50:7710–6.
- [113] Powell GJ, Southby J, Danks JA, Stillwell RG, Hayman JA, Henderson MA, et al. Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared with other sites. *Cancer Res* 1991;51:3059–61.
- [114] Henderson M, Danks J, Moseley J, Slavin J, Harris T, McKinlay M, et al. Parathyroid hormone-related protein production by breast cancers, improved survival, and reduced bone metastases. *J Natl Cancer Inst* 2001;93:234–7.
- [115] Vargas SJ, Gillespie MT, Powell GJ, Southby J, Danks JA, Moseley JM, et al. Localization of parathyroid hormone-related protein mRNA expression in breast cancer and metastatic lesions by *in situ* hybridization. *J Bone Miner Res* 1992;7:971–9.
- [116] Cho YM, Lewis DA, Koltz PF, Richard V, Gocken TA, Rosol TJ, et al. Regulation of parathyroid hormone-related protein gene expression by epidermal growth factor-family ligands in primary human keratinocytes. *J Endocrinol* 2004;181:179–90.
- [117] Lorch G, Gilmore JL, Koltz PF, Gonterman RM, Laughner R, Lewis DA, et al. Inhibition of epidermal growth factor receptor signalling reduces hypercalcemia induced by human lung squamous-cell carcinoma in athymic mice. *Br J Cancer* 2007;97:183–93.
- [118] Richard V, Rosol TJ, Foley J. PTHrP gene expression in cancer: do all paths lead to Ets? *Crit Rev Eukaryot Gene Expr* 2005;15:115–32.

- [119] Gilmore JL, Scott JA, Bouizar Z, Robling A, Pitfield SE, Riese 2nd DJ, et al. Amphiregulin-EGFR signaling regulates PTHrP gene expression in breast cancer cells. *Breast Cancer Res Treat* 2007;110:493–505.
- [120] Lu X, Wang Q, Hu G, Van Poznak C, Fleisher M, Reiss M, et al. ADAMTS1 and MMP1 proteolytically engage EGF-like ligands in an osteolytic signaling cascade for bone metastasis. *Genes Dev* 2009;23:1882–94.
- [121] MacLeod JR, Yano S, Chattopadhyay N, Brown EM. Extracellular calcium-sensing receptor transactivates the epidermal growth factor receptor by a triple-membrane-spanning signaling mechanism. *Biochem Biophys Res Commun* 2004;320:455–60.
- [122] Gschwind A, Hart S, Fischer OM, Ullrich A. TACE cleavage of proamphiregulin regulates GPCR-induced proliferation and motility of cancer cells. *EMBO J* 2003;22:2411–21.
- [123] Normanno N, Morabito A, De Luca A, Piccirillo MC, Gallo M, Maiello MR, et al. Target-based therapies in breast cancer: current status and future perspectives. *Endocr Relat Cancer* 2009;16:675–702.
- [124] Albain KS, Elledge R, Gralishar WJ, Hayes DF, Rowinsky E, Hudis C, et al. Open-label, phase II, multicenter trial of ZD1839 ('Iressa') in patients with advanced breast cancer. *Breast Cancer Res Treat* 2002;76:S33.
- [125] von Minckwitz G, Jonat W, Fasching P, du Bois A, Kleeberg U, Luck HJ, et al. A multicentre phase II study on gefitinib in taxane- and anthracycline-pretreated metastatic breast cancer. *Breast Cancer Res Treat* 2005;89:165–72.
- [126] Cristofanilli M, Valero V, Mangalik A, Royce M, Rabinowitz I, Arena FP, et al. Phase II, randomized trial to compare anastrozole combined with gefitinib or placebo in postmenopausal women with hormone receptor-positive metastatic breast cancer. *Clin Cancer Res* 2010;16:1904–14.
- [127] Leary AF, Drury S, Detre S, Pancholi S, Lykkesfeldt AE, Martin IA, et al. Lapatinib restores hormone sensitivity with differential effects on estrogen receptor signaling in cell models of human epidermal growth factor receptor 2-negative breast cancer with acquired endocrine resistance. *Clin Cancer Res* 2010;16:1486–97.
- [128] Johnston S, Pippen Jr J, Pivot X, Lichinitser M, Sadeghi S, Dieras V, et al. Lapatinib combined with letrozole versus letrozole and placebo as first-line therapy for postmenopausal hormone receptor-positive metastatic breast cancer. *J Clin Oncol* 2009;27:5538–46.